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ABSTRACT

NEURONAL ANALOGUES OF
CONDITIONING PARADIGMS
Roy Emerson Clymer III

It has been suggested that there is a neuronal basis of conditioning that occurs at the synapse between two cells and that the firing of the postsynaptic cell is required for conditioning to occur. Neurophysiological investigations of learning have relied heavily on studies of the marine mollusk Aplysia californica because of its large, hardy, identifiable neurons. Results from these studies have not clearly determined whether electrophysiological procedures analogous to conditioning paradigms cause changes which could constitute the basis of in vivo conditioning.

This study examined the conditionability of cells in the abdominal ganglion of the Aplysia californica in an attempt to replicate, clarify and extend previous results. It also investigated the role of postsynaptic cell firing in producing the observed conditioning.

Individual cells within the ganglion were recorded from using intracellular recording techniques. Mild electrical stimulation of the inputs to the cell produced a postsynaptic potential. The postsynaptic potential was repeatedly paired with one of four forms of

stimulation. In the "conditioning" procedure, the postsynaptic potential was followed in 300 milliseconds by repeated firing of the cell induced by intense stimulation of additional inputs to the cell. In the "pseudoconditioning" procedure, the stimulation induced firing followed the postsynaptic potential by 10 seconds. In the "clamp" procedure, the cell was hyperpolarized by current injection throughout the procedure. This prevented cell firing in response to the intense stimulation which was presented as in the conditioning procedure. In the "current injection" procedure, the postsynaptic potential was followed in 300 milliseconds by repeated cell firings induced by injection of depolarizing current into the cell. The four procedures were given in counterbalanced orders.

Following the conditioning and clamp procedures, the postsynaptic potential was significantly larger. This was not true of the pseudoconditioning procedure. The current injection procedure resulted in a significant decrease in the size of the postsynaptic potential. These results were interpreted to rule out a role for postsynaptic cell firing in producing conditioning.

Cells were classified according to their response to the conditioning and pseudoconditioning procedures. Cells which did not respond to the conditioning procedure were called unconditionable. If they responded to both the conditioning and the pseudoconditioning procedure, they were called pseudoconditionable. If they responded to the conditioning procedure but not the pseudoconditioning procedure,

they were labeled truly conditionable. Pseudoconditionable cells were more responsive to the clamp procedure than were the other two cell types. This suggested that the mechanism of pseudoconditioning differed from that of true conditioning.

The pseudoconditioning procedure was shown to have an inhibitory effect upon an immediately following conditioning procedure. This appeared to be directly analogous to the conditioned inhibition observed in studies of conditioning in whole animals.

The electrophysiological procedures produced neuronal results which were directly analogous to results produced by *in vivo* conditioning experiments. Although postsynaptic cell firing does not appear to play a role in the production of this conditioning, the mechanism which produces the changes remains unclear. Whatever the mechanism, however, sophisticated information processing appears to occur in simple neuronal systems.

NEURONAL ANALOGUES OF

CONDITIONING PARADIGMS

by

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Background

The study of learning is fundamental to psychology. Experimental investigations of behavioral changes caused by experience constitute a vast body of data and a number of different theories of learning have been proposed. In spite of the existence of some very comprehensive and powerful theories (see Hilgard and Bowers, 1966, for a thorough exposition), none has gained wide, general acceptance. The field appears to have adopted Skinner's (1950) functional approach to the analysis of learning. This approach has provided a great deal of information about when and under what conditions learning occurs. It has not revealed, however, how learning occurs. Recent advances in the fields of neurophysiology and neuroanatomy have stimulated renewed interest in neuronal models of learning. It may now be possible to explain learning in terms of the underlying events occuring within the neurons that make up an animal's nervous system.

Neurophysiologists have developed a fairly complete explanation of the processes involved in neural activity and nervous system signaling. The inside of a neuron has a negative electric charge with respect to the surrounding body fluids. This electric potential which develops across the cell's outer membrane can vary due to intrinsic or extrinsic influences. Whenever it decreases past some threshold value, an automatic process may be initiated which generates

a temporary, self-propogating reversal of the transmembrane electric potential. This "action potential" spreads throughout the neuron's distant processes and may eventually activate muscles or glands or signal other neurons.

Typical neuro-neuronal connections consist of a "leader" cell which transmits electrical impulses to a "follower" cell. The site at which the leader interacts with the follower is called the synapse. The synapse is not a physical connection between neurons. Instead, a small space separates them. The firing of the presynaptic neurons causes the release of a chemical, the neurotransmitter, into the synaptic cleft. The neurotransmitter diffuses across the cleft where it activates receptors in the postsynaptic neural membrane. The neurotransmitter may be excitatory and cause the membrane potential to become less negative (depolarize) and to move toward the firing threshold. Alternatively, the neurotransmitter may have an inhibitory effect at the particular synapse and move the postsynaptic neuron away from the firing threshold by making the membrane potential more negative in a process called hyperpolarization. Increases and decreases in the excitability of the neuron may be produced by other mechanisms as well. Also some mechanisms exist which can cause a depolarization yet at the same time inhibit cell firing. Classically, however, cell firing is the consequence of sufficient depolarization.

Although the mechanisms of interneuronal communication have been well established, the changes underlying most forms of learning

have thus far eluded investigators. That something in the nervous system changes as a result of experience and is the source of subsequent changed behavior is accepted by many scientists. Because of the chemical mechanism of transmission, the synapse has been the focus of attempts to explain the neuronal basis of learning. If the learning process somehow alters the amount of neurotransmitter released or the responsiveness of the postsynaptic receptors, then this might be the basis of learning.

Such a mechanism of learning might work as follows: suppose that a particular external stimulus causes a particular neuron (or group of neurons) to fire, i.e., generate an action potential. Suppose further that the firing of this neuron results in the release of an excitatory neurotransmitter onto the receptors of a motor neuron controlling some behavior of interest. If presentation of the external stimulus fails to elicit the behavior, it may be because only a small amount of transmitter was released, or, alternatively, the postsynaptic receptor is not very sensitive to the transmitter. Suppose finally that some sequence of external stimuli and behaviors results in learning and presentation of the external stimulus now elicits the behavior. If the presynaptic neuron now releases a substantially greater amount of neurotransmitter into the synaptic cleft when it fires, then this changed efficacy of the synapse could be the neuronal basis of learning. Alternatively, the change in synaptic efficacy could occur postsynaptically. The presynaptic terminal may release the same amount of neurotransmitter but the

postsynaptic depolarization might be larger due to some other mechanism. A great deal of experimental and theoretical work has attempted to determine if and when synapses change their efficacy.

The Hebbian Synapse

Since its introduction in 1949 by D. O. Hebb, the concept of a synapse which is modified as a result of presynaptic and postsynaptic electrical activity has played an important role in theoretical and experimental investigations into the neuronal basis of learning. Hebb noted that many features of a particular form of learning, associative learning, could be explained by postulating that a synapse increases in efficacy if the presynaptic cell firing is closely followed by postsynaptic cell firing. Associative learning refers to a form of learning in which an association (i.e., a related occurrence) develops between an external stimulus and a behavior. There are two simple forms of associative learning, classical and operant conditioning.

Classical conditioning refers to the form of learning first described by Pavlov (1927) in his experiments on the salivation response of dogs. In this form of conditioning an unconditioned stimulus (UCS) reliably elicits a reflexive response called the unconditioned response (UCR). In Pavlov's experiments meat powder was the UCS which elicted salivation, the UCR. Another stimulus, the conditioned stimulus (CS) initially fails to elicit the UCR. If the CS is repeatedly presented just before the UCS, however, eventually

the CS, when presented alone, will elict a response nearly identical to the UCR. This response is called the conditioned response (CR). In Pavlov's experiments a bell was frequently employed as the CS, and the dogs came to salivate at the sound of the bell after it had been repeatedly paired with the meat powder.

The other form of associative learning, operant conditioning, was named by Skinner (1938), based on his experiments with pigeons and other animals. In operant conditioning the investigator (or the natural environment) presents a stimulus contingent upon the animal's emission of a particular behavior (called an operant). If presentation of the contingent stimulus results in an increase in the frequency of the operant's emission, the stimulus is called a reinforcer. If the operant decreases in frequency, the stimulus is called a punisher. The presence of another stimulus, the discriminative stimulus, may indicate when a particular contingency is in effect. For example, a green light in a chamber might indicate when a rat's bar-press would be rewarded with a food pellet. Skinner observed that in the natural environment a particular behavior emitted in the presence of certain stimuli reliably leads to punishing or rewarding consequences and hence he believed that operant conditioning was a fundamental mode of learning.

Hebb noted that in both forms of learning a stimulus situation which initially played no part in the control of behavior came to be associated with the occurrence of the behavior. In classical

conditioning the CS comes to elicit the CR while in operant conditioning the behavior comes to be emitted in the presence of the discriminative stimulus. Hebb posited that at the neuronal level, the emission of the behavior could correspond to the firing of a particular (postsynaptic) neuron while the presentation (or presence) of the CS (or discriminative stimulus) could generate the firing of a neuron presynaptic to the one involved in the emission of behavior. He noted further that given such a relationship between neurons, the sequence of events involved in conditioning would correspond to repeated paired firings of the presynaptic and postsynaptic neurons. He, therefore, hypothesized that if a neuron A repeatedly participates in the firing of cell B, then the efficacy of neuron A as one of the cells causing the firing of cell B would increase with repeated pairings of the firing of cells A and B. He suggested that this change could be brought about by a corresponding change in the functional efficacy of the syanpse between cells A and B. By postulating a functionally modifiable synapse which changed efficacy as a result of conditions directly analogous to those occurring in learning, Hebb seemed to have provided a simple yet powerful theory of the neuronal events which might underlie conditioning.

Hebb's proposal (and variations thereof) has been widely used by theorists from a variety of disciplines to construct models of neuronal processes in order to provide explanations of various learning or behavioral phenomena. For example, it is well known that light projected on the retina causes the rods and cones to fire. The

pattern of firing is precisely duplicated in portions of the visual cortex. Similarly, stimulation of sensory neurons on the body surface precisely generates the firing of particular neurons in the somato-sensory cortex, which leads to a "mapping" of the body sensors onto the cortex. In addition, there is a similar mapping of the motor cortex to effector muscles throughout the body. Stimulation of a certain portion of cortex reliably leads to the activation of a particular muscle. All of these mappings are thought to require learning for their development. Models of this process using Hebbian synapse have been developed by Takeuchi and Amari (1979).

In another example, Hubel and Weisel (1962) discovered that certain neurons of the visual cortex respond selectively to the orientation of the images in the visual field. Some neurons fire when a vertical bar of light is presented, others respond to a horizontal bar, while still others respond to various diagonal orientations. They showed that this selectivity develops through experience and can be altered if an animal has one or both eyes masked during development. Bienenstock, Cooper and Munro (1982) used a variation of a Hebbian synapse in a simple neuronal network which convincingly modeled the results obtained by Hubel and Weisel.

One of the most common uses of the Hebbian formulation has been in the development of theories of adaptive elements. Adaptive elements are theoretical information processing constructs. Networks of adaptive elements are intended to replicate the known capabilities

of biological neurons and/or nervous systems. Starting with simple rules about the processing and modification of signals, the models derive information about the capabilities of complex neuronal networks. An excellent review of previous work in the field, plus an innovative and powerful new model, is provided by Sutton and Barto (1981). It is important to note, however, that all the models they review postulate some form of modified output of the postsynaptic unit as a necessary precondition for changed synaptic efficacy. That is, in all these models, synaptic change is presumed to occur if and only if presynaptic firing is correlated with either postsynaptic cell firing or a change in the firing frequency of the postsynaptic cell. This requirement of a role for altered postsynaptic activity in changing synaptic efficacy has come to be the definition of a Hebbian synapse.

Possible Hebbian Synapses

In spite of its wide utility in model building, there is very little research which provides supporting evidence for a Hebbian form of synaptic modulation (Little and Shaw, 1975). In fact, in a recent article taken from a series of lectures honoring the work of Hebb, Goddard (1980) cites only one example of a synapse modified by postsynpatic activity, the synapses of the fascia dentata of the hippocampus. Goddard cites evidence from his and other laboratories which shows that two apparently independent effects are observed if axons of the perforant pathways to the fascia dentata are stimulated.

First, a mild tetanizing (quickly repeated) stimulus yields a short-term potentiation which is similar to the post-tetanic potentiation (PTP) seen at the neuromuscular junction and in some other neurons. This effect is fairly short-lived, decaying back to pre-stimulation levels within 5 to 10 minutes. Second, in addition to PTP, there exists a long-term change in responsiveness to stimuli which has come to be called "enhancement" (Bliss and Lomo, 1973). This long lasting increase in synaptic transmission may persist up to 16 hours. McNaughton (1978) found that PTP and enhancement can coexist in the same neuronal system. Although the details of the parametric features which determine and distinguish PTP and enhancement have not been fully worked out, there does seem to be at least one significant difference between the conditions which elicit the two phenomena. Enhancement appears to require the stimulation of a certain minimum number of synapses, whereas PTP will occur at even a single stimulated synapse. Goddard also states that the threshold number of inputs necessary for enhancement is quite close to the number required to cause postsynaptic cell firing. Thus, the process of enhancement is compatible with the operation of a Hebbian mechanism.

There are, however, important differences between what is observed in the hippocampus and Hebb's original conception. Evidence from Douglas (1978a, 1978b) and McNaughton (1978) shows that if the postsynaptic cell firing is blocked by stimulation of an inhibitory input at the appropriate time, enhancement still occurs. Although

this result is substantially different from Hebb's formulation, Goddard argues that the associative process could still result from "cooperative action of convergent inputs" (Goddard, 1980, p. 236). He suggests that a synapse will be enhanced if it fires concurrently with a sufficient number of other synapses. In other words, postsynaptic cell firing (or a change in postsynaptic firing frequency) is not necessary.

An interesting and possibly conflicting result was obtained by Thompson (1976) as a result of his investigations into the classical conditioning of the eye-blink reflex of the nictitating membrane (NM) in the rabbit. As part of his investigation into the neuronal basis of the development of a conditioned response, he investigated the influence of a potential conditioned stimulus, a tone, on the "excitability" of the NM response. Thompson stimulated the abducens motor neurons via extracellular current injection which elicited an eye-blink. In this procedure an electrode is placed in the vicinity of the abducens motor neurons and a voltage is applied to the electrode which excites nearby neurons and causes them to fire. By noting the extent of the NM response as a function of the applied current, Thompson showed that these neurons directly controlled the NM response. In order to assess the effect of the tone CS on the excitability of these motor neurons, he paired presentation of the CS with sub-maximal current injections at various inter-stimulus intervals (ISIs). Increased excitability was indicated by a response of the NM which was larger than that produced by the current injection

alone (which had been chosen to produce a minimal response).

Thompson found that the CS produced a change in the excitability of the NM response which was a non-linear function of the ISI. Short ISIs (less than 50 milliseconds) caused no increase in excitability. Above 50 milliseconds, the response increased rapidly, peaked at approximately 500 milliseconds and then declined rapidly so that ISIs of more than a second or two produced virtually no conditioning. Thompson suggests that this result implies that a population of neurons activated by the CS makes an excitatory synapse upon the abducens motor neurons. Further, and of more importance here, Thompson noted that in the course of these excitability experiments which involved paired presentations of the CS and current injections at various ISIs, a conditioned response to the CS was observed to develop. Presentation of the CS alone eventually led to an enhanced NM response. Conditioning developed even though no external stimulus acting as a UCS was presented. This finding is contrary to traditional notions of classical conditioning which demand a UCS. Yet here, conditioning occurred even though no UCS was present.

These results seem to suggest that conditioning occurred at the level of the abducens motor neurons and, further, that such conditioning may have been produced by a Hebbian mechanism. The excitability data led Thompson to conclude that the CS fires a population of neurons which terminate upon and excite the abducens

motor neurons. The conditions of the experiment resulted in the firing of these presynaptic neurons just before the abducens motor neurons were fired via extracellular current injection. Over the course of the experiment this paired firing resulted in the development of the firing of the abducens motor neurons in response to presentations of the CS alone. This apparent conditioning could have been the result of an increase in the efficacy of the synapse between the presynaptic and postsynaptic neurons. Such an increase is precisely what would be expected if a modifiable synapse of the type postulated by Hebb existed between the sensory neurons and the abducens motor neurons.

These experiments do not permit a firm conclusion that a change in the postsynaptic activity was a necessary condition for the apparent change in synaptic efficacy. Although this is the simplest explanation, two caveats prevent its adoption. First, we do not know the full extent of the neuronal population which was stimulated by the CS. Neither do we know if the CS does, in fact, stimulate neurons which directly synapse upon the abducens motor neurons. Although the physiological evidence strongly suggests that the CS excites a population of neurons which make excitatory synapse upon the abducens motor neurons, anatomical evidence is completely lacking. Second, because the abducens motor neurons were stimulated with extracellular, rather than intracellular, current injections, it is possible that neurons (or axons) other than the abducens motor neurons were stimulated. This raises the possibility that another mechanism,

presynaptic facilitation, may be responsible for the observed conditioning.

Presynaptic Facilitation

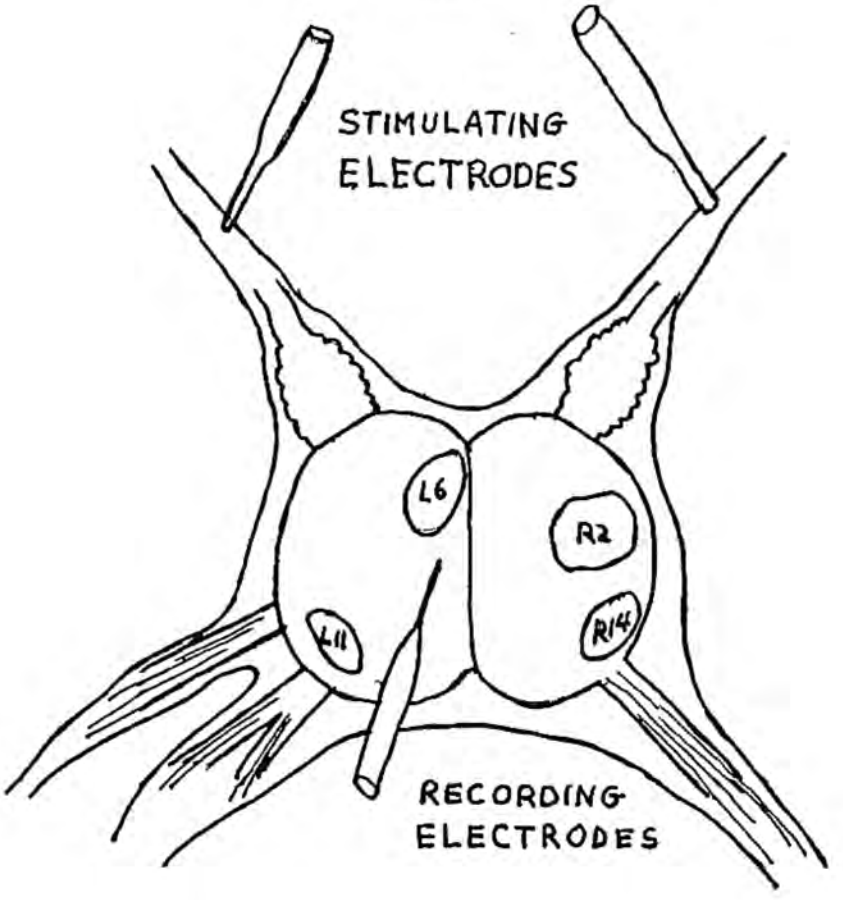
In presynaptic facilitation, a third, facilitating neuron makes an axo-axonal synapse upon the presynaptic terminal of a leader-follower pair. Firing of the facilitating neuron leads to the release of a modulatory neurotransmitter onto the presynaptic terminal. This neurotransmitter does not necessarilly cause a change in the membrane potential of the postsynaptic cell, although it may. Instead, it alters the characteristics of the presynaptic terminal so that subsequent firings of the presynaptic neuron will result in an increased release of transmitter and, therefore, an enhanced response in the postsynaptic neuron. This mechanism has been proposed by Kandel as an explanation of a variety of learning phenomena including dishabituation (Kandel, 1979), classical conditioning (Kandel and Tauc, 1965b) and associative processes in general (Kandel, 1979).

In an early investigation into the cellular basis of learning, Kandel and Tauc (1965a, b) conducted a series of experiments on cells of the abdominal ganglion of the European sea hare, Aplysia depilans. (See Appendix 1 for a summary description of the Aplysia and its nervous system.) The ganglion were dissected out and placed in a perfusion chamber. Suction stimulating electrodes were attached to the left and right connectives or, occasionally, to others of the

three peripheral nerves (see Figure 1). After a cell had been impaled with an intracellular recording electrode, the current in one of the stimulating electrodes on one of the connectives was adjusted so as to produce a small excitatory postsynaptic potential (EPSP) in the impaled cell. This was called the "test stimulus." The current in the electrode attached to the other connective was increased to the point at which it produced repetitive firing of the impaled cell. This was called the "priming stimulus." The question of interest was whether the repeated pairing of these two stimuli would constitute a neuronal analogue of conditioning by producing an increased EPSP in response to the test stimulus alone. If so, then the larger EPSP would be more likely to cause the postsynaptic cell to fire. Presuming that the firing of the postsynaptic cell is related to the emission of behavior, this would mean that an electrophysiological procedure analogous to _in vivo_ conditioning procedures would have produced a change in synaptic efficacy which could form the basis of the changed behavior observed in learning.

Kandel and Tauc administered the test stimulus at a constant rate (one stimulation every 10 seconds) to produce an EPSP of constant magnitude. The test stimulus was then paired 20 or more times with the priming stimulus. The priming stimulus followed the test stimulus with an ISI of 300 milliseconds. In most cells, this pairing produced "no conditioning," i.e., there was no increase in the magnitude of the EPSP produced by the test stimulus after conditioning as compared to before. In 15 of 90 unidentified cells located near the midline of

FIGURE 1



DORSAL VIEW OF ABDOMINAL GANGLION OF THE APLYSIA SHOWING STIMULATING AND RECORDING ELECTRODES (AFTER KANDEL AND TAUC, 1965a).

the ganglion, however, this pairing produced conditioning; presentation of the test stimulus resulted in the production of a markedly increased EPSP (an average 100%). In one identified cell, R2, the giant cell found in the right upper quadrant of the ganglion, this pairing also produced conditioning. Kandel and Tauc observed, however, that when the priming stimulus was presented alone, an enhanced response still developed in R2. Because pairing of the test and priming stimuli was not required to produce this enhanced response, the enhancement was called "pseudo" conditioning to distinguish it from the "true" conditioning which was observed to occur in other cells. In these cells, conditioning was produced only when the two stimuli were paired and not when they were applied alone.

Kandel and Tauc (1965b) also performed a series of experiments designed to investigate the mechanism of the observed conditioning. They chose to perform these on the R2 cell of the Aplysia because it could be readily identified from preparation to preparation. Thus, although R2 demonstrated only pseudoconditioning, because of its identifiability, it was deemed a suitable choice to investigate the mechanism of conditioning. In order to determine if a Hebbian mechanism of conditioning produced the observed facilitation, Kandel and Tauc (1965b) investigated the possible role of the firing of the postsynaptic cell. They were able to do this by pairing the test stimulus with three different forms of the priming stimulus, each of which had different effects on the firing of the postsynaptic cell.

Kandel and Tauc established the conditionability of R2 by first using a priming stimulus which consisted of a rapid sequence of five stimulations of the siphon nerve which reliably produced five action potentials in R2. Again, whether paired with the test stimulus or not, this priming stimulus resulted in facilitation of the test response. In a second procedure, they directly initiated five action potentials in R2 by injecting depolarizing current directly into the cell. This depolarized the cell past its firing threshold and caused it to fire. This "current injection" procedure was also paired with the test stimulus but Kandel and Tauc found that it failed to produce conditioning. Their third procedure consisted of stimulating the siphon nerve with the priming stimulus as before, but at the same time hyperpolarizing the cell by injecting negative current into it. This prevented the cell from firing in response to the priming stimulus. They found that when the test stimulus was paired with the priming stimulus in this "clamped" cell, conditioning still occurred.

From these results, Kandel and Tauc (1965b) excluded the firing of the postsynaptic cell as a requirement for facilitation. Having ruled out a role for the postsynaptic action potential, they argued that these results suggest that presynaptic facilitation may be the mechanism of synaptic modification responsible for producing the observed pseudoconditioning.

The inability of a postsynaptic action potential to generate conditioning was further demonstrated by Wurtz, Castellucci and

Nusrala (1967). They tried to produce conditioning in single cells using a simpler procedure than did Kandel and Tauc. Wurtz et al. (1967) investigated neurons in the abdominal ganglion of the Aplysia californica, the Pacific variety of the sea hare. Unlike Kandel and Tauc, they did not remove the ganglion, but left it intact within the animal while recording. This enabled them to observe the production of numerous spontaneous postsynaptic potentials produced by the intact afferent nerves. They recorded from various unspecified cells with a recording/stimulating electrode. The output of the electrode was connected to a computer which was programmed to recognize and categorize the spontaneously generated EPSPs. That is, individual EPSPs were recognized on the basis of their rise times, duration, and maximum amplitude. After the computer program had successfully differentiated an EPSP, the identified EPSP was paired with cell firing produced by current injection. Since animal conditioning experiments have shown conditioning to be dependent upon the interval between the CS and the UCS, the interval between the EPSP and the subsequent cell firing was varied in different cells. In addition, the number of pairings was varied, with a maximum of several hundred pairings performed upon some cells. The pairing of an EPSP and a cell firing was repeated in 44 cells.

According to Hebb's hypothesis, this pairing of the EPSP and postsynaptic cell firing should produce an increase in the efficacy of the synapse and a resulting increase in the magnitude of the EPSP. In the experiment by Wurtz and his colleagues (1967), the EPSPs were

produced by the firing of presynaptic neurons. After an identified EPSP occurred, presumably produced by a specific presynaptic terminal, the cell was fired via direct current injection. This would seem to constitute a reasonable operationalization of what Hebb meant by cell A "participating" in the firing of cell B. If so, and if Hebb were correct, an increase in the efficacy of the synapse between the two cells should have been produced by the pairings in the experiment by Wurtz et al. (1967). Such an increase in efficacy might be indicated by an increased frequency of action potentials in the postsynaptic cell. The enhanced EPSPs could be expected to result more frequently in an action potential in the postsynaptic cell. In order to determine whether or not the pairings resulted in an increased frequency of postsynaptic cell firings, Wurtz and his colleagues computed the percentage of time an identified EPSP was followed within a certain time period by an action potential. They then compared these percentages before and after pairing the EPSP with induced postsynaptic cell firings. (The time interval after the EPSP within which they looked for an action potential was usually 100 milliseconds, but other intervals from 0 to 1000 milliseconds were also examined.) In only six of 44 cells did the pairing produce an increase in the percentage of times the EPSP was followed by a subsequent action potential. In five of these six cells there was also an increase in the frequency of action potentials following identified EPSPs which had NOT been paired with induced cell firings. The increased frequency of action potentials which did occur was not specific to the pairing of the EPSP and cell firing. Wurtz and his

collegues therefore attributed the increase to some change in the postsynaptic cell membrane which affected the EPSPs non-specifically. They concluded that this experiment had failed to show an effect of postsynaptic cell firing on synaptic efficacy.

It is possible that any increase in efficacy produced by the pairings in experiment by Wurtz et al. (1967) was too small to result in the generation of an action potential. A small increase in synaptic efficacy, however, might show up as an increase in the amplitude of the EPSP. This possibility was also examined by Wurtz and his colleagues. In a second series of experiments, they produced EPSPs in the impaled cell by stimulating the afferent nerves. These evoked EPSPs were then paired with cell firing produced by current injection or antidromic stimulation of the cell. In 35 cells, Wurtz et al. (1967) report that no increase in amplitude following pairing was observed in any cell. They reported that no conditioning occurred even when a variety of parameters were manipulated including the number of pairings, the duration of the experiment, and the ISI.

The results of Wurtz et al. (1967) and Kandel and Tauc (1965a, b) seem to preclude any role for postsynaptic cell firing in mechanisms of synaptic modulation because repeated pairings of presynaptic and postsynaptic cell firings consistently failed to result in conditioning. These findings, therefore, seriously challenge Hebb's hypothesis. Besides a Hebbian mechanism, presynaptic facilitation is probably the most viable alternative explanation for

associative learning because it is compatible with the available evidence. It is an acceptable explanation of the results obtained by Kandel and Tauc when they conditioned R2 cells with three different priming stimuli. Presynaptic facilitation is not ruled out by the negative results of Wurtz et al. (1967) and Kandel and Tauc (1965a, b) concerning the effect of a postsynaptic action potential since presynaptic facilitation does not require postsynaptic cell firing. As a general mechanism of conditioning, presynaptic facilitation seems to require that a facilitative neuron make axo-axonal synapse upon every synapse capable of modification. This conclusion is based on the very nature of the proposed mechanism because it specifies that a particular synapse is modified through the action of a facilitative neuron which makes axo-axonal synapse upon the modifiable presynaptic terminal. If learning is the result of a process of synaptic modification, a great number of such axo-axonal synapses would have to exist within the brain in order to account for our tremendous learning capabilities. Anatomical evidence, however, does not seem to indicate a widespread distribution of axo-axonal synapses (Bullock, 1977).

In addition to the anatomical evidence arguing against presynaptic facilitation, there is an unanswered question about the conditions under which the facilitating neuron causes a change in synaptic efficacy: Even if a facilitating neuron does make an axo-axonal connection upon a given synapse, what conditions cause the proper facilitating neuron to fire and cause the appropriate synapse to be modified? Suppose it is the UCS which causes the facilitating

neuron to fire and hence causes facilitation to take place, as has been proposed by Kandel and Schwartz (1982). Because a given CS could be paired with almost any number of responses, does the UCS producing each response also result in the firing of a separate facilitating neuron? If so, this would imply that not only does the UCS activate the motor neuron controlling the emission of the UCR, but it must also activate the facilitative neuron. Further, this facilitative neuron must then make an axo-axonal synapse upon the terminals of the leader neuron which synapse upon the motor neuron and are activated by any potential conditioned stimulus. The complicated structure required to actualize these parallel but functionally separate effects of the UCS would be difficult to reconcile with results indicating that conditioning can occur in preparations as simple as the spinal cords of cats (Woody and Brozek, 1969).

As a general model of associative conditioning, presynaptic facilitation has even greater difficulties accounting for operant conditioning. In operant conditioning there is no identifiable UCS which elicits the behavior. What then activates the facilitative neuron and produces conditioning? It is possible that it is reinforcement which activates the facilitating neuron. Reinforcement, however, occurs considerably after the behavior which produced it. How, then, would the reinforcing stimulus activate the specific facilitative neuron which would modify only the synapse between neurons responsive to the preceding sensory environment and the preceding behavior? These questions are not easily answered in a way

that allows for a simple explanation of the diverse learning phenomena known to psychologists. Therefore, the utility of presynaptic facilitation as a general model of associative conditioning, as has been proposed by Kandel (Kandel and Tauc, 1965 b, Kandel, 1979, Kandel and Schwartz, 1982), is questionable.

A Modified Hebbian Synapse

Kandel and Tauc's (1965b) results may provide support for a modified Hebbian mechanism suggested by Goddard (1980). In their experiments, Kandel and Tauc showed that although pseudoconditioning of R2 was not produced when the test stimulus was paired with cell firing induced by intracellular current injection, it was produced when a large EPSP which was generated by a lesser test stimulus and hyperpolarization of the postsynaptic cell was used as the priming stimulus. As noted earlier, Goddard (1980) suggested that enhancement might be the result of the generation of a sufficiently large EPSP in the cell due to the stimulation of a sufficient number of inputs. According to this hypothesis, Goddard's results may be explained as follows: When a large number of the axons of the perforant pathway are stimulated, the firing of each presynaptic terminal occurs contemporaneously with the large EPSP produced by the simultaneous firing of the other stimulated presynaptic terminals. Thus, each synapse is eligible for modification, its efficacy is increased, and the observed enhancement results. If lesser numbers of the presynaptic terminals are stimulated, the subsequent summed EPSP is

smaller than the EPSP necessary to cause a modification of synaptic efficacy and hence no enhancement is observed.

If the magnitude of the EPSP needed to produce conditioning is roughly equal to that necessary to produce an action potential in the postsynaptic cell, the changes in synaptic efficacy will be produced by events which lead to postsynaptic cell firing. That is, the large EPSP which might modify a particular eligible synapse will also result in the firing of the postsynaptic cell. This leads to a result functionally quite similar to Hebb's hypothesis without the requirement of actual postsynaptic cell firing. Such a mechanism, if demonstrated, would be consistent with the fundamental feature of a Hebbian synapse which has been so useful to model makers: the specificity of conditioning to the occurrence of an output.

The provisional acceptance of the idea that conditioning may be produced by the pairing of presynaptic cell firing with a sufficiently large EPSP is hindered by the fact that R2, the neuron of the abdominal ganglion investigated by Kandel and Tauc (1965a, b), demonstrated pseudoconditioning and not the true conditioning observed in other neurons of the ganglion. In the experiment by Kandel and Tauc (1965b), R2 demonstrated conditioning when the test stimulus was paired with a large EPSP produced when the cell was hyperpolarized. The fact that a large EPSP paired with the test EPSP in R2 did lead to conditioning cannot be generalized to cells which displayed true conditioning. Because two procedures lead to two results,

pseudoconditioning and true conditioning may be the results of separate mechanisms. It is not clear whether the results observed in R2 generalize to cells displaying true conditioning. Therefore, although the results of Kandel and Tauc are consonant with a modified Hebbian mechanism, it is uncertain whether they extend to truly conditionable cells.

There is good reason to suspect that there may be a difference between neuronal mechanisms of true conditioning and the mechanism of the observed "pseudoconditioning" of R2 and other cells. Conditioning experiments with whole organisms show a similar difference between true conditioning and enhanced responses produced by presentation of the UCS alone. Indeed, in conditioning experiments, it is necessary to rule out a possible sensitizing effect of the UCS which may appear to be conditioning (Thompson, 1976). Since sensitization and true conditioning coexist and can be separated at the behavioral level, it is important to try to separate them at the neuronal level as well. Sensitization would appear as pseudoconditioning at the neuronal level, i.e., increased responding which occurs when a priming stimulus is given unpaired with an EPSP. In order to accurately determine the neuronal mechanism of true conditioning, true conditioning must be distinguished from processes such as sensitization which might yield similar results. Only then will it be possible to distinguish between presynaptic facilitation, a Hebbian mechanism, or a modified Hebbian mechanism of true conditioning.

It is premature to conclude that the mechanism of conditioning is known even for pseudoconditionable cells. Kandel and Tauc (1965a, b) generalized their findings to rule out any role for an action potential in the postsynaptic cell in producing the conditioning observed in various cells of the abdominal ganglion. Their claim was based primarily on their investigations of R2, a cell which displayed pseudoconditioning rather than true conditioning. In addition, R2 is so large that it is extremely difficult to prevent its firing via injection of hyperpolarizing current. Its size also makes it difficult to fire with depolarizing current injection. Before conclusions are drawn regarding the role of postsynaptic cell firing in conditioning, it seems advisable to investigate this question in conditionable cells other than R2.

The results of Kandel and Tauc (1965a,b) are not the only evidence bearing on this issue. The study by Wurtz et al. (1967) also investigated the R2 cell of the Aplysia. They paired the firing of R2 with an EPSP produced by electrical stimulation of a connective and, like Kandel and Tauc, they report no conditioning. They also investigated a total of 35 unidentified cells of the abdominal ganglion using a test stimulus paired with cell firings induced with intracellular current stimulation and reported that none of them showed any evidence of conditioning. No attempt was made to determine whether the 35 cells were conditionable by any other procedure. Since the cells were randomly selected, it is possible that all 35 of the cells Wurtz and his colleagues investigated (other than R2) were, in

fact, not conditionable in the first place. Since Kandel and Tauc (1965a) found that only 15 of the 90 unidentified cells they investigated showed conditioning of any kind, this possibility may exist. The evidence does not clearly rule out a role for the postsynaptic action potential in the production of true conditioning.

Further evidence on the conditionability of cells via current injection does not seem to be available. Only the article by Wurtz et al. (1967) presents any experimental evidence relevant to this point. In a review of various neuronal underpinnings of behavioral phenomena, Kandel and Spencer (1968) mention only the Wurtz et al. (1967) article in support of the failure of a postsynaptic action potential to induce conditioning. More recent evidence does not seem to be available. Although the articles discussed here are frequently cited according to the Science Citation Index, none of the articles citing either Kandel and Tauc (1965a, b) or Wurtz et al. (1967) provided any experimental evidence relevent to these issues.

Because of the questions concerning the firing of R2 and the fact that only R2 was investigated, it seems premature to decide that an action potential in the postsynaptic cell plays no role in pseudoconditioning. It would seem desirable to investigate a number of other cells showing pseudoconditioning before making a judgment about the role of the postsynaptic action potential in pseudoconditioning. Even if the results for R2 were unambiguous, supporting evidence from other pseudoconditionable cells would still

be desirable.

Before questions concerning the mechanisms of true and pseudoconditioning can be answered, it appears that the definitions of these cell types need additional clarification. The Kandel and Tauc (1965a) investigation reported that out of the 90 unidentified cells they investigated, 75 showed "no conditioning" in response to the paired test and priming stimuli. A control for the specificity of conditioning to pairing was conducted in only 5 of the 15 which showed conditioning. All 5 showed no conditioning with unpaired presentations of the priming stimuli and therefore appeared to be truly conditionable. Whether the other 10 conditionable cells are truly or pseudoconditionable is unknown. In addition, no data from the 75 "unconditionable" cells are presented in the article and thus it is unclear how much, if any, conditioning these cells displayed. This is all the more important since no explicit definition of what constitutes conditionability was offered by Kandel and Tauc. No cell listed as conditionable by Kandel and Tauc (1965a, p. 7) shows less than a 40% facilitation after conditioning. Without the data from the "unconditionable cells", however, it is unclear whether this reflects a characteristic of the distribution.

In conclusion, although the literature offers some interesting information concerning the existence and mechanisms of a neuronal analogue of conditioning, several issues remain unclear. Are there distinct populations of unconditionable, pseudoconditionable and truly

conditionable cells? In conditionable cells, what role, if any, does postsynaptic cell firing play in the production of conditionability? Is this different between pseudo and truly conditionable cells? Even in one of the most studied neurophysiological preparations, the abdominal ganglion of the Aplysia, there is no certainty about the answers to these questions.

OVERVIEW OF STUDY

The present study was designed to answer two questions. 1. Using electrophysiological procedures analogous to whole animal conditioning paradigms, can distinct populations of unconditionable, pseudoconditionable, and truly conditionable cells be identified? 2. If so, what role, if any, does postsynaptic cell firing play in producing conditioning in pseudo- and/or truly conditionable cells? These questions were addressed in a replication and extension of the procedures employed by Kandel and Tauc (1965a, b). The population of cells used in this study were chosen to match those investigated by Kandel and Tauc in the initial part of their experiment (1965a). In addition three of the four procedures performed upon the cells in this experiment were exact replications of the procedures performed by Kandel and Tauc (1965a, b). The fourth procedure, pseudoconditioning, was a variant of one they performed.

While the procedures employed in this study were quite similar to those of Kandel and Tauc, the order of their implementation was quite different. In their initial investigation, after a healthy cell was penetrated, the test stimulus was paired with the priming stimulus with an interval of 300 milliseconds between the two. If, after the pairing, the cell showed no enhanced response to the test stimulus, they went on to another cell. On only 5 of the 15 which did show a response to pairing was a control for the specificity of pairing performed. On only one cell, R2, was a test of the mechanism

of conditioning performed. (The tests, however, were performed on this cell in many preparations.) In this study four procedures were performed upon every cell (when possible). This allowed each cell to be classifed as unconditionable, pseudoconditionable, or truly conditionable depending upon its response to two of the procedures. Further, the two other procedures provided information about the role of the postsynaptic cell firing in producing any observed conditioning.

On each viable cell four procedures were performed. Chart 1 provides a summary description of the sequence of events in the four procedures. Between the administration of each procedure there was a three to five minute rest period so that any conditioning produced by a previous procedure would have time to decay before the subsequent procedure was performed. In one procedure, called "conditioning," the test stimulus was followed by the priming stimulus after an interval of 300 milliseconds. In this procedure, as well as the other three procedures, the two stimuli were presented together 10 times. This procedure was intended to replicate the sequence of events and timing which is frequently found to be effective in producing conditioning in whole organisms (Thompson, 1976). An ISI of 300 milliseconds approaches the optimal interval for producing conditioning (Thompson, 1976; Kandel and Tauc, 1965a).

In order to ascertain whether any observed conditioning was specific to the pairing of the test and priming stimuli, a control

CHART 1

SEQUENCE OF EVENTS	PROCEDURE			
	CONDITIONING	PSEUDOCOND.	CLAMP	CUR. INJECTION
CELL HYPER-POLARIZED?	NO	NO	YES	NO
INITIAL MEMBRANE POTENTIAL	-50	-50	-110	-50
TRIGGER TEST STIMULUS?	YES	YES	YES	YES
INTERVAL BETWEEN TEST AND POTENT STIMULUS	300 MSEC.	10 SEC.	300 MSEC.	300 MSEC.
SOURCE OF POTENT STIMULUS	STIMULATE CONNECTIVE	STIMULATE CONNECTIVE	STIMULATE CONNECTIVE	INJECT DEPOLARIZING CURRENT
POSTSYNAPTIC CELL FIRING?	YES	YES	NO	YES
CONDITIONING EXPECTED?	YES	NO	?	?

procedure called "pseudoconditioning" was performed. In the pseudoconditioning procedure, the priming stimulus followed the test stimulus by 10 seconds; that is, it occurred in the middle of the interval between presentations of the test stimulus (which is presented continuously every 20 seconds throughout the experiment). Although still paired sequentially with the test stimulus, in the pseudoconditioning procedure the priming stimulus occurred outside the window of time normally effective in producing conditioning. Although there is some debate about this issue, it is generally accepted that, with the exception of a very few specific reflexes such as taste aversion, ISIs greater than 1 to 2 seconds do not produce conditioning (Thompson, 1976; Schwartz, 1978). By having a 10 second ISI in the pseudoconditioning procedure, it was possible to determine whether or not any observed conditioning was the result of the sensitizing effect of presentation of the priming stimulus alone.

In order to investigate the mechanism of conditioning and the role of postsynaptic cell firing in producing conditioning, two additional procedures were performed on each cell. One, the "clamp" procedure consisted of pairing the test and priming stimuli with an inter-stimulus interval of 300 milliseconds while preventing the firing of the cell by injecting hyperpolarizing current during the procedure. Cells were extensively hyperpolarized via current injection and allowed to stabilize. Then, the test stimulus was followed (after 300 milliseconds) by the priming stimulus. Again, the two stimuli were paired 10 times. By preventing the postsynaptic cell

from firing during this procedure, any observed conditioning must be attributed to a mechanism other than that which relies on the occurrence of a postsynaptic action potential.

Finally, the "current injection," procedure consisted of the direct injection of depolarizing current into the cell for a duration of 1 second. Current injection commenced 300 milliseconds after the test stimulus and the magnitude was adjusted so as to produce roughly the same number of action potentials as that produced by the priming stimulus. This provided a method of firing the cell independent of the priming stimulus and enabled a comparison between the enhancement produced by either the conditioning or pseudoconditioning procedures and the enhancement produced by directly firing the postsynaptic cell. Thus, the role of the postsynaptic cell firing in producing any observed conditioning could be determined.

To reiterate, the four procedures were: 1. Conditioning, in which the cell was fired by stimulating a connective 300 milliseconds after the test stimulus. 2. Pseudoconditioning in which the priming stimulus followed the test stimulus by 10 seconds. 3. Clamp where the cell was hyperpolarized and then the stimuli sequence of the conditioning procedure was executed. 4. Current injection where the cell was fired via depolarizing current injection 300 milliseconds after the test stimulus.

Performing each procedure on every cell permitted both a replication and extension of previous work. The results of the conditioning procedure provide almost an exact replication of Kandel and Tauc's (1965a) survey of the conditionability of the cells of the Aplysia's abdominal ganglion. Similarly, the current injection procedure is almost an exact replication of one portion of the investigation of Wurtz et al. (1967) regarding the effect of cell firing on conditionability. Further, because the four procedures were performed upon the abdominal giant cell of many ganglia, Kandel and Tauc's (1965b) investigation of the mechanism of the pseudoconditioning of R2 was also replicated.

Although each of these "replications" was embedded in the larger design and, hence, was not an exact replication, the comparability of data was assured by the fact that the four procedures were presented in a counterbalanced order. Thus, although it is possible that the pseudoconditioning procedure may affect the results of the conditioning procedure, for example, counterbalancing precluded this as an explanation of any differences between these results and previous work. This same counterbalancing also allowed a less clouded classification of cells. In the Kandel and Tauc (1965a) experiment, the test for specifity of pairing, when performed, was always performed after the conditioning procedure. The possibility that the conditioning procedure had an effect upon subsequent procedures hampers the interpretation of the Kandel and Tauc results. Finally, counterbalancing the procedures enabled the direct investigation of

the possible effects of one procedure upon another.

The fact that all four procedures were performed upon each cell extended previous work in a number of ways. First, it provided clearer, less ambiguous classification of cells as either pseudo-, true, or unconditionable because the conditioning and pseudoconditioning procedures were performed upon every cell (when possible). This increased the probablity that the classification was representatative and not merely of a small number of random cells. Moreover, the method of designation of cells as conditionable first employed by Kandel and Tauc (1965a) was clarified. This functional classification enabled an extension of the results concerning one pseudoconditionable cell (R2) to a larger population of pseudoconditionable cells. In addition, it permitted investigation of the effects of the clamp and current injection procedures themselves by performing them on unconditionable cells. Finally, and most importantly, the functional classification allowed the investigation into whether or not postsynaptic cell firing plays a different role in producing conditioning in pseudo- vs. truly conditionable cells.

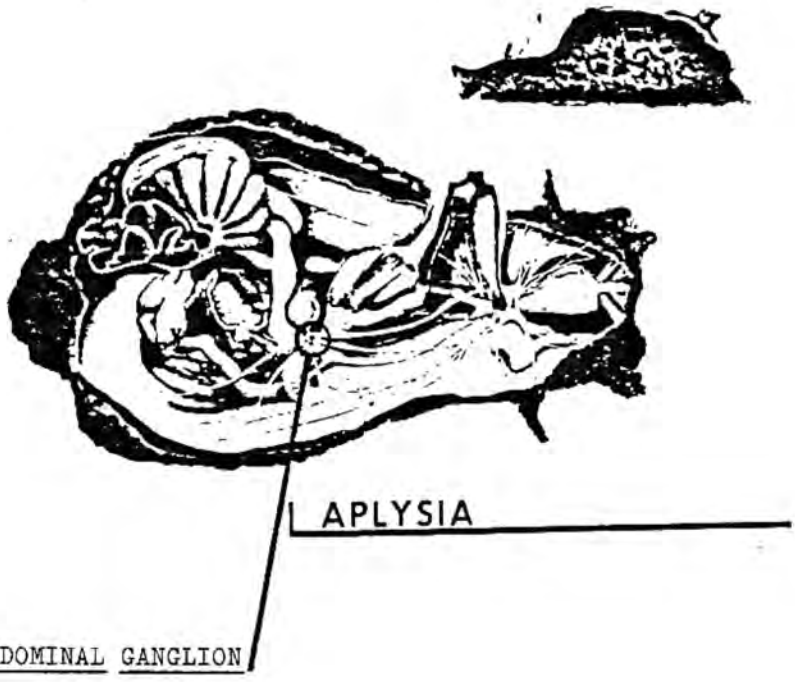
METHODS

Subjects

The experimental preparation was the abdominal ganglion obtained from the marine mollusk, Aplysia californica (the Pacific sea hare). This variety was chosen because of it ready availability (see Appendix 1). Animals were obtained from Pacific Biomarine in LaJolla, California.

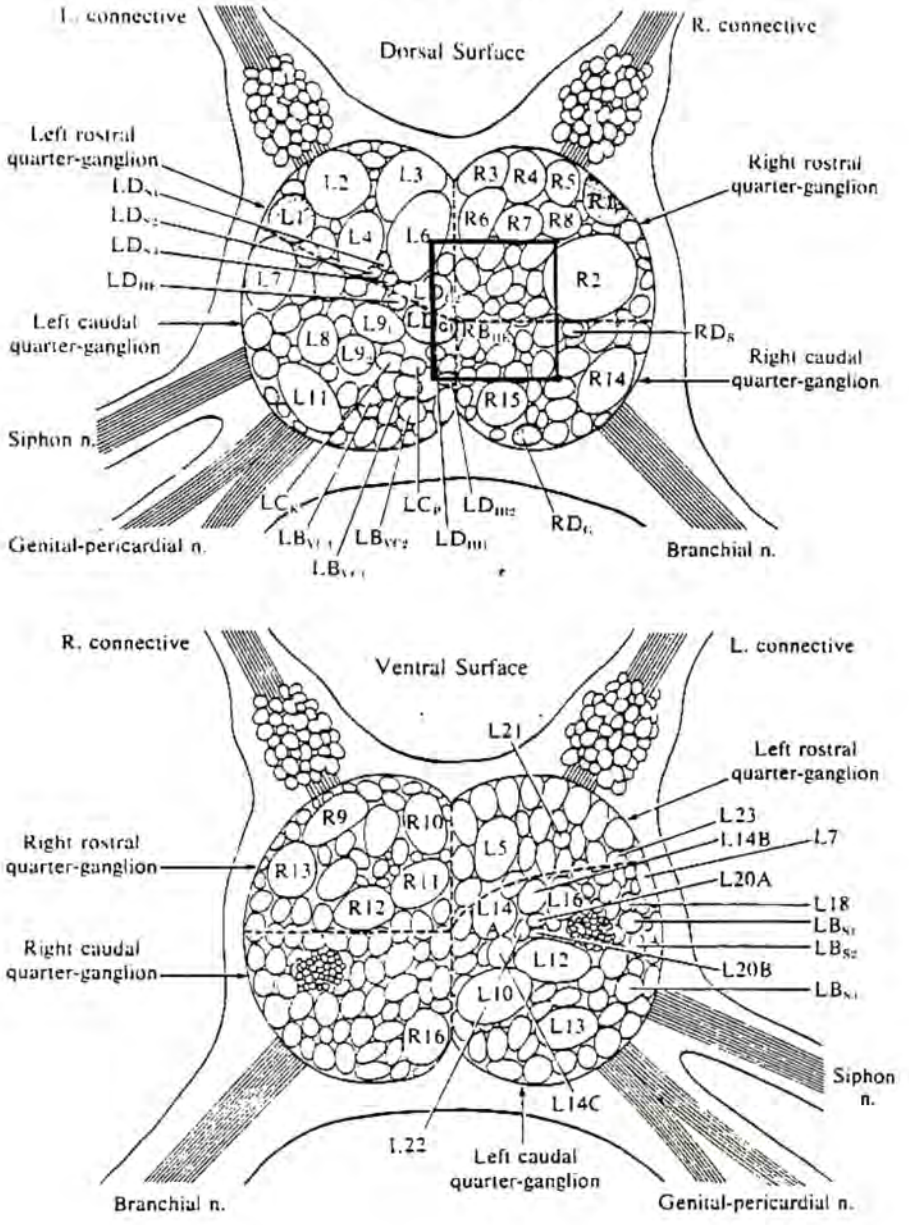
The Aplysia is the preferred preparation for a variety of reasons. Because of the simplicity of the nervous system, a great many individual neurons have been identified and neuronal circuits subserving overt behaviors have been mapped out (Kandel, 1979). Although the nervous system is rudimentary, the animal is capable of various forms of learning, including classical conditioning (Carew, Hawkins, and Kandel, 1983). Individual neurons are large and hardy and hence can be easily impaled with one or more electrodes and can be expected to survive for the several hours this experimentation requires. Finally, because of the Aplysia's rudimentary circulatory system, cells require no special perfusate to maintain viability and can therefore be supported by the perfusion of inexpensive, laboratory-made, artificial sea water. Figure 2 is a lateral view of a dissected Aplysia and indicates the location of the abdominal ganglion. Figure 3 shows a schematic view of the dorsal and ventral surfaces of the ganglion, indicating the position of various

FIGURE 2



DRAWING OF DISSECTED APLYSIA SHOWING LOCATION OF ABDOMINAL GANGLION.

FIGURE 3



DORSAL AND VENTRAL SURFACES OF ABDOMINAL GANGLION INDICATING LOCATIONS OF SOME IDENTIFIED CELLS. THE BOX OUTLINES THE PRIMARY AREA OF INVESTIGATION (FROM KANDEL, 1976).

identified cells. The box in the ventral view outlines the area Kandel and Tauc (1965a) reported as that within which they found most of their conditionable cells. Cells within this area were the primary target of the present investigation as well, although other cells were studied.

Intracellular Recording Equipment

Membrane potentials were measured with a Dagan Model 8100 single electrode voltage clamp electrometer operating in current clamp mode. This amplifier is a low gain, high impedance, differential amplifier with negative capacitance feedback. It has the capability of passing current through the recording electrode into the cell by switching at a frequency of 3 kilohertz between recording from the cell and injecting current. This allows simultaneous recording and current injection into the cell. Recordings were made on a Gould 4-channel strip chart recorder. One channel monitored the test and priming stimulus current pulses delivered to the left and right connectives. A second channel monitored current injected into the cell by the amplifier. This included relatively constant hyperpolarizing current used to maintain the cell at a constant membrane potential during a procedure as well as depolarizing current pulses which fired the cell. The third and fourth channels monitored the cell's membrane potential, one channel at 10 millivolts per division, the other at 2 millivolts per division. The membrane potential was also displayed on a Tektronic dual channel differential

oscilloscope. One channel displayed a low gain indication of the membrane potential so its actual value could be read. The other channel produced a high gain display on which postsynaptic potentials could be observed.

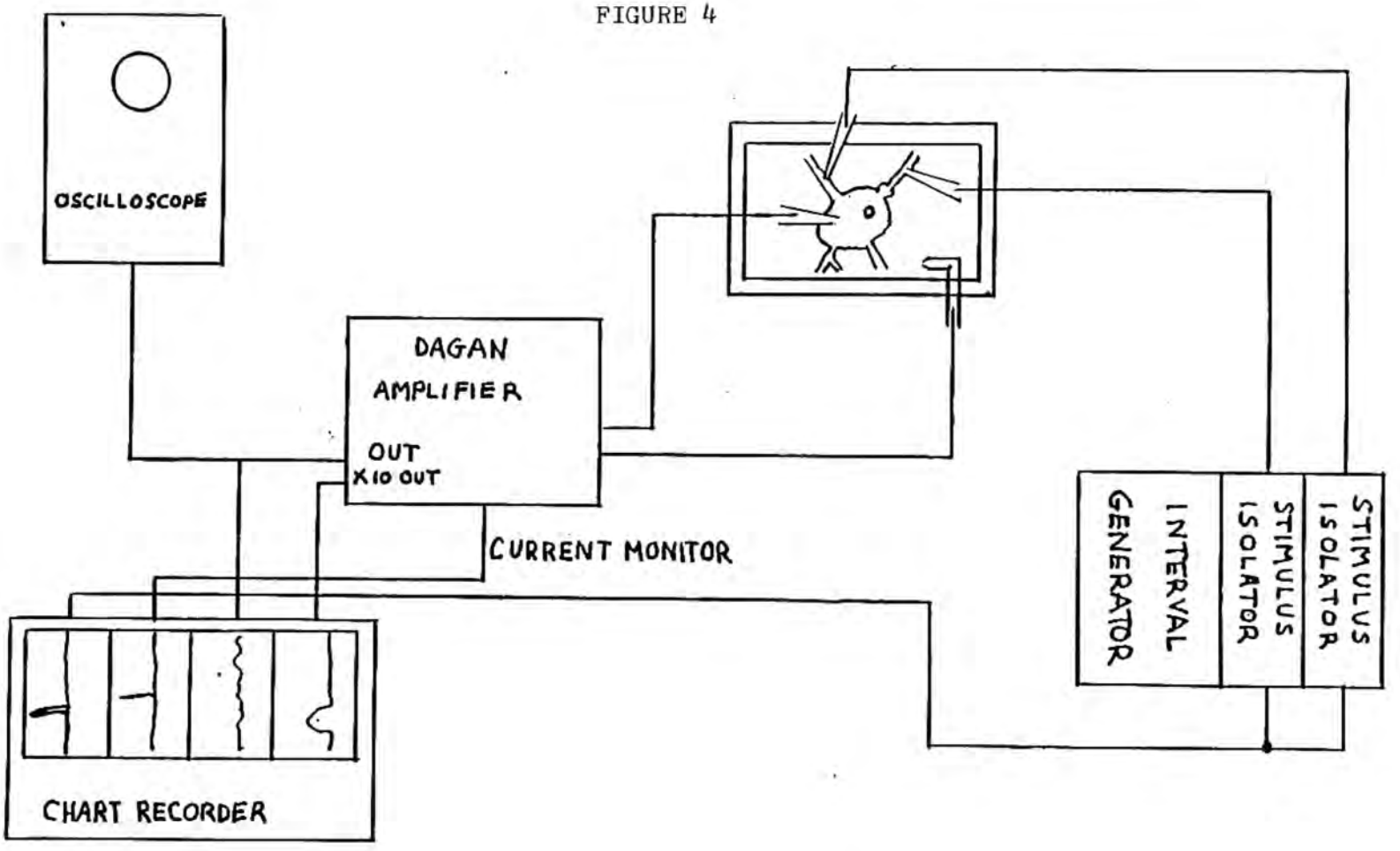
The timing of the presentations of the various stimuli was controlled by a system consisting of a WPI interval generator, three WPI 850a Stimulus Isolators, three 831 Pulse Modules and an event counter. This system allowed precise control of the amplitude, duration, and timing of intracellular current injections, the stimuli to the connectives, the ISI, and the number of trials performed. Figure 4 is a block diagram of the eletrical components of the recording and stimulating setup.

Electrodes

Recording electrodes were pulled from four inch long boro-silicate glass pipettes with a 1.2 millimeter outside diameter using a David Kopf Instruments Model 700C vertical pipette puller. Tip diameters of one-half micron or less producing resistances of less than 5 megohms were used. They were filled with 2 molar potassium acetate and inserted into WPI silver/silver chloride half-cells filled with 3 molar potassium chloride.

Suction electrodes were constructed from the same boro-silicate glass as the recording elctrodes. They were handpulled,

FIGURE 4



SCHEMATIC DIAGRAM OF RECORDING SET UP

sanded, and polished to provide tip diameters of approximately 0.5 millimeters. They were filled with artifical sea water, as were the WPI half-cells into which they were inserted. The half-cells had ports which were connected to spring-loaded syringes. These provided the suction which attached the stimulating electrodes to the left and right connectives.

Procedures

An Aplysia was first pinned out, ventral side up, to a wax surface or cork board. A longitudinal incision extending from the tail to the head was made in the base (ventral surface). The body wall was pinned laterally to expose the contents of the hemocoel including the nervous system. The abdominal ganglion, and approximately 2 centimeters of the left and right connectives, were dissected out of the Aplysia and pinned out dorsal surface upright in a cooled perfusion chamber whose bottom was coated with Sylguard (Dow Corning Co., Midland, MI.). The ganglion was completely submerged in a circulating bath of artificial sea water. The formula for artificial sea water is given in Appendix 2. The perfusion chamber and the sea water were cooled by a circulating, thermostatically controlled cooling system which maintained a temperature of 17 degrees Celsius.

After the ganglion was pinned out, suction electrodes were attached to the left and right connectives. The protective capsule

covering the cells of the ganglion was then cut, exposing individual cells which were then impaled with the recording/stimulating electrode. Only cells meeting the following conventional criteria of healthy Aplysia cells were used: 1. The resting membrane potential must have been at least as negative as -30 millivolts. 2. The cell must have shown a membrane resistance (measured by current injection) of at least 1 megohm. If a cell met these criteria, a slight amount of hyperpolarizing current was injected (approximately 3 nanoamps) and the cell was allowed to stabilize for at least 15 minutes.

After allowing a 30 to 45 minute interval for the cells to recover normal functioning following these initial procedures, the effects of stimulating the connectives were examined to see whether or not suitable test and priming stimuli could be obtained. It was necessary that the stimulation of one connective produce a sufficiently large EPSP that it could be used as a priming stimulus (i.e., that it could be made to cause the cell to fire). At the same time, stimulation of the other connective must have produced either an excitatory or inhibitory PSP to be used as the test stimulus. If stimulation produced suitable results, the stimulus strengths were adjusted so that the stimulated connective producing the largest response in the cell was designated the priming stimulus while the other connective provided the test stimulus. The test response was elicited by a 100 microsecond pulse of sufficient magnitude to elicit a reliably repeated, readily identifiable PSP in the cell. The priming stimulus consisted of a sequence of 6 one-millisecond pulses

separated by 150 milliseconds. The priming stimulus current intensity was adjusted so that each pulse produced an action potential in the cell.

The PSPSs produced by the test stimulus were not necessarily monosynaptic. Stimulation of the connective may have fired one or more interneurons whose firing ultimately produced the PSP observed in the cell. Also the PSP could be a composite response, consisting of the response generated by several stimulated input pathways. In spite of these limatations, changes in the size of the PSP would still reflect the changes in synaptic functioning of interest.

At this point the cell was a candidate for study. The test stimulus was activated and repeated at 20 second intervals. Before the first procedure was performed, however, an additional 15 minute period was allowed to pass to enable the cell to finally stabilize. If, at the end of that period, the test stimulus still produced a constant PSP, the first procedure was conducted.

The four procedures were administered in counterbalanced orders. Four procedures can occur in 1 of 24 orders. The order of the procedures performed upon a particular cell was determined by randomly choosing the first procedure, then the second, etc. If a particular order had already been performed, then a different one was randomly selected. If a cell survived the first procedure of the order, then for the next cell a new order was chosen. If it did not

survive the first procedure, that same order was used for the next cell until at least the first procedure of the order had been satisfactorily performed. Each order was performed completely on one cell before a second cell was assigned a previously performed order. Appendix 3 shows the 24 orders and the algorithm used to select the procedures.

Some aspects of the four procedures were identical regardless of the type of procedure. The recording chart was sped up so that detailed recordings could be obtained of the PSPs produced by the three test stimuli prior to the procedure. After the procedure, detailed recordings of the three test responses following the procedure were obtained. Three to 5 minutes were permitted to elapse after one procedure ended before the next procedure was started in order to allow any facilitation caused by a prevous procedure to decay before conducting a subsequent procedure (Kandel and Tauc, 1965a).

The four procedures were performed as follows: In the conditioning procedure the test stimulus was followed in 300 milliseconds by the priming stimulus. After 10 instances of the priming stimulus, it was discontinued. This sequence produced 10 paired presentations of the test and priming stimuli with an intertrial interval of 20 seconds and an ISI of 300 milliseconds. The number of trials, ISI, strength of the priming stimulus, and intertrial interval were all chosen to replicate as precisely as possible the conditions of Kandel and Tauc's (1965a, b) experiments.

In the pseudoconditioning procedure, the parameters were precisely the same as in the conditioning procedure except that the priming stimulus was given 10 seconds after the test stimulus; thus, it occurred in the middle of the interval between test stimuli. The priming stimulus was presented 10 times. This control for the specificity of pairing was different from that employed by Kandel and Tauc (1965a, b). They presented the priming stimulus alone at 20 second intervals but with the test stimulus turned off. They noted that turning off the test stimulus for a period resulted in a temporarily enhanced response to the test stimulus when it was resumed. This complication seemed to make such a control procedure undesirable. In addition, in in vivo animal conditioning experiments, not providing the UCS is considered an unacceptable control procedure (Rescorla and Wagner, 1972). For these reasons, the above procedure was substituted for that of Kandel and Tauc's.

The conditioning and pseudoconditioning procedures differ only in terms of the interval between the test and primng stmulus. The 10 second ISI used in the pseudoconditioning procedure is outside the realm of ISIs usually found effective in producing conditioning in whole orgaims and may therefore constitute an effecive control for the sensitizing effects of the priming stimulus. Nonetheless, the conditionability of neurons as a function of the ISI is unknown, and differences from in vivo conditioning may be expected.

The clamp procedure was parametrically identical to the conditioning procedure with one exception: The cell was first hyperpolarized via current injection. After the preceding procedure, hyperpolarizing current was injected into the cell through the recording electrode by the Dagan single electrode clamp operating in the switched current-clamp mode. The amplifier switched at a frequency of 3 kilohertz between reading the membrane potential and injecting current into the cell. Sufficient current was injected into the cell to bring its membrane potential to approximately -100 to -120 millivolts. The cell was then allowed to stabilize at this membrane potential for approximately five minutes before the procedure was begun. The chart speed was then increased to record the response to three sequential test stimuli. Following this, the priming stimulus was presented 10 times paired with the test stimulus with a 300 milliseconds ISI. Then the responses to the three test stimuli after the procedure were recorded. This completed the procedure, and the hyperpolarizing current was turned off, allowing the cell to return to its normal resting potential. Sometimes the return to resting potential produced repetitive firing of the cell. Usually this ceased after a brief period and the next procedure was performed. If it persisted, however, the experiment was discontinued and another cell was investigated.

The current injection procedure was different from the others in that the left or right connective was not stimulated to produce the priming stimulus. In the current injection procedure, commencing 300

milliseconds after the test stimulus, a one-second pulse of depolarizing current was injected into the cell. This current pulse would cause the cell to fire, and the amount of current injected was adjusted so as to produce 4 to 8 action potentials during the pulse. The intent was to mimic the firing pattern produced by the priming stimulus but to do so by action potentials directly initiated by the drop in membrane potential produced by the depolarizing current.

One procedure must have been successfully completed in order to go on to another. If a procedure damaged a cell, the experiment was halted. Such damage might be evidenced by a drop in the membrane potential to below the least acceptable value (-30 millivolts) or a failure to produce a PSP in response to the test stimulus. In addition, if the cell failed to fire in response to the priming stimulus or current injection during a procedure, the results were not included. If the cell did fire in response to the priming stimulus during the clamp procedure, either the cell was additionally hyperpolarized or the cell was not included. If a procedure was repeated because it was unsuccessful the first time, the cell was not classified as one in which one of the 24 orders had been successfully performed.

ANALYSIS

Scoring

Scoring of the records was performed as follows: The magnitude of the PSP produced by the test stimulus was determined by the change in the membrane potential from the time of the initiation of the test pulse to the point of maximum excursion. In quiet cells, the resting potential was quite constant before a test stimulus. The constant variability of the membrane potential in some cells, however, made necessary a more arbitrary method of designating the membrane potential. In these cells the base level was taken to be the value of the membrane potential at the instant of initiation of the test stimulus. The magnitude was measured as the distance from this point to the point of maximum excursion of the PSP.

The amplitude of the PSPs were measured by pen excursion on the chart recorder. Three PSPs preceding each procedure and three PSPs following the procedure were measured for comparison. The measurements were analyzed by a computer program that averaged the three readings and converted them to a single value (in millivolts) which represented the averaged size of the PSP before or after the procedure. These pre-values and post-values were useful in determining the effect of a single procedure; however they had certain limitations. The magnitude of the PSP produced by the test stimulus varied from cell to cell. In addition, it was usually markedly larger

during the clamp procedure than during the other procedures (due to the greater driving force in the hyperpolarized cell). This variability made it impossible to compare changes in the absolute magnitude of the PSP. In order to control for the variability in the size of the PSP, percent change scores were calculated. The percent change was calculated as the difference between the post-procedure and pre-procedure values of the PSP divided by the pre-procedure value.

Statistical procedures

Comparisons of group means between two groups were performed using paired t-tests. Comparisons between more than two groups used a repeated measures analysis of variance.

RESULTS

A total of 113 abdominal ganglia were investigated. In these ganglia, 109 cells met all the criteria of viability, responded adequately to the test and priming stimuli, and had one or more procedures successfully performed upon them. In two cells the stimulation of the left and right connectives was reversed so that the former priming stimulus became a test stimulus and vice versa. This yielded data on two additional cases for a total of 111 cases meeting all criteria. Of the 109 cells, R2 was investigated 8 times, RB cells or suspected RB cells 16 times, L7 and L9 one time each, and the remaining 85 were of unknown identity.

The procedures were not all successfully completed upon all cells, and some procedures were performed more than once upon a single cell. This resulted in the conditioning procedure being performed 119 times, the pseudoconditioning control procedure 97 times, the clamp procedure 99 times and the current injection procedure 103 times. Table 1 shows the number of cells upon which each procedure was performed one or more times. The conditioning procedure was the most frequently repeated since it potentially revealed the most information about a cell.

Figure 5 reproduces a typical record from the high gain channel of the chart recorder. First, it shows the three excitatory postsynaptic potentials produced by the test stimulus before the

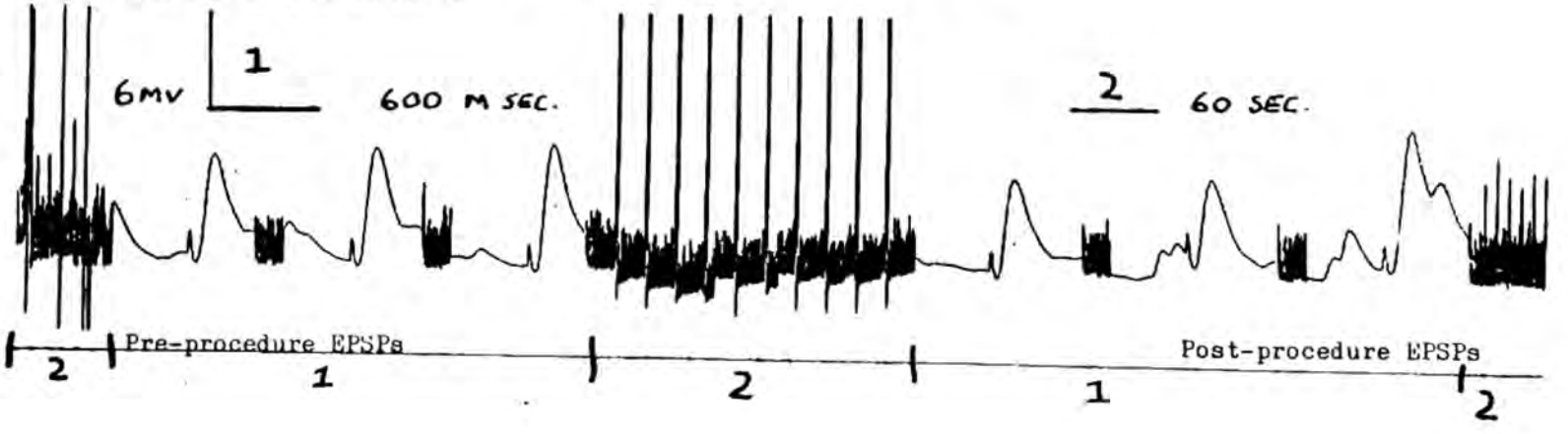
TABLE 1

NUMBER OF TIMES THE PROCEDURES WERE PERFORMED

PROCEDURE	PERFORMED ONCE UPON	PERFORMED TWICE UPON	PERFORMED 3 TIMES UPON	TOTAL INSTANCES
CONDITIONING	92 CELLS	25 CELLS	2 CELLS	119
PSEUDO-CONDITIONING	89	8	0	97
CLAMP	93	6	0	99
CURRENT INJECTION	97	6	0	103

FIGURE 5

CHART RECORDING SHOWING HIGH GAIN RECORD OF A CURRENT INJECTION PROCEDURE. THREE POSTSYNAPTIC POTENTIALS PRECEDE AND FOLLOW THE ACTION POTENTIALS INDUCED BY 10 OCCASIONS OF DEPOLARIZING CURRENT INJECTION. THE TIME SCALE ON THE RIGHT APPLIES TO THE DARKER AREAS WHERE THE CHART IS SLOWED BETWEEN THE TEST STIMULI AND DURING THE PAIRING OF THE STIMULI.



procedure. These are recorded at a fast chart speed (25 millimeters per second) so as to reveal details. The chart is slowed to .025 millimeters per second during the procedure (in this case, current injection), and in between test stimuli. The 10 spikes are the action potentials induced by the ten occasions of pairing the test stimulus with current injection. Afterwards, there are again the three postsynaptic potentials generated by the test stimulus alone. In this case the average magnitude of the three pre-procedural PSPs was 7.6 millivolts while the post-procedure average was 7.5. The record also shows the typical variation in the magnitude of the response to the test stimulus. Recordings from the other three procedures would look virtually identical except that the priming stimuli in the pseudoconditioning procedure occur midway between two test stimuli.

The postsynaptic potential produced by the test stimulus was excitatory in all cases but one. This was partially the result of the selection criteria which required that the priming stimulus be capable of firing the cell. In only one case was a cell found which responded with an inhibitory postsynaptic potential to stimulation of one connective while at the same time responding with a sufficiently large excitatory postsynaptic potential to stimulation of the other connective so as to induce postsynaptic cell firing.

The Effects of the Procedures

Table 2 shows the mean PSP before and after each procedure.

TABLE 2

MEAN POSTSYNAPTIC POTENTIAL (IN MILLIVOLTS)
BEFORE AND AFTER THE PROCEDURES

VARIABLE	MEAN	MINIMUM	MAXIMUM	VARIANCE
BEFORE CONDITIONING	4.74	.50	19.7	12.49
AFTER CONDITIONING	5.13	.40	16.9	11.03
BEFORE PSEUDO-CONDITIONING	5.09	.50	17.7	11.67
AFTER PSEUDO-CONDITIONING	5.21	.30	20.0	11.80
BEFORE CLAMP	6.85	.10	22.4	21.69
AFTER CLAMP	7.26	.10	18.9	21.70
BEFORE CURRENT INJECTION	5.00	.30	23.1	14.53
AFTER CURRENT INJECTION	4.43	.30	16.8	11.64

Data from every instance a procedure was performed are included. The values are in millivolts. (It should be remembered that each observation was itself the average of three postsynaptic potentials.) The various procedures produced approximately a 5 millivolt PSP with variance of 10 to 20 millivolts.

An initial question is: Does a given procedure produce a change in the magnitude of the response to the test stimulus? Standard electrophysiological technique would answer this on a cell by cell basis. This was not done for two reasons. First, comparing across cells allows for the use of more powerful statistical techniques. Second, usual technique calls for each cell to serve as its own control with a return to baseline measurement taken an appropriate time interval after each procedure. This method would add considerably to the time it took to perform a given procedure. Given the complications of attempting to perform at least four potentially damaging procedures on each cell, it was deemed advisable to forgo this method.

Another approach to this question which averages the response across cells is to perform a paired t-test of the pre-procedure and post-procedure means. The results of such an analysis are presented in Table 3. In this table data from every procedure that was performed is included in the analysis. It therefore includes cases where the same procedure was repeated two or more times upon a cell and cases where the four procedures were not performed sucessfully

TABLE 3

COMPARISONS OF THE MEAN POSTSYNAPTIC POTENTIAL
BEFORE AND AFTER EACH PROCEDURE
USING ALL INSTANCES OF EACH PROCEDURE

PAIRS	N	MEAN	DEVIATION	t	df	p (2 tailed)
BEFORE CONDITIONING		4.74	3.53			
	119			2.01	118	0.047
AFTER CONDITIONING		5.13	3.32			
BEFORE PSEUDO-CONDITIONING		5.09	3.42			
	97			0.61	96	0.55
AFTER PSEUDO-CONDITIONING		5.21	3.44			
BEFORE CLAMP		6.85	4.68			
	99			1.72	98	0.089
AFTER CLAMP		7.26	4.66			
BEFORE CURRENT INJECTION		5.00	3.81			
	103			4.51	102	0.000
AFTER CURRENT INJECTION		4.43	3.41			

upon a cell. The table indicates that the mean PSP after the conditioning procedure was significantly larger than the mean PSP before the procedure. The pseudoconditioning procedure, in which the priming stimulus was presented with a 10 second inter-stimulus interval, however, did not result in a significant increase in responsiveness.

Kandel and Tauc's results would lead to an expectation that the clamp procedure would yield an increase in responsiveness. Table 3 shows that that expectation was confirmed with a one-tailed probability of just less than .05. Finally, the current injection procedure resulted in a significant decrease in response to the test stimulus. It is interesting to note that the value before conditioning does not differ significantly from the value before current injection. This seems to indicate that the initial state of the cells was the same at the begining of the two procedures.

The higher mean postsynaptic potentials occurring in the clamp procedure are an effect of the hyperpolarization of the cell. The PSP is increased due to the greater "driving force" at the more negative membrane potential. This fact precluded direct comparisons of the PSPs between procedures.

The above results were computed using every instance of a procedure, even if it was performed on a cell more than once. This presents the possibility that the cases in which a procedure was

repeated violate the assumption of independence underlying the t-test. Therefore, another analysis was conducted which used only the first instance of each procedure on a cell. Thus, there were no repeated instances of a given procedure on a single cell. Table 4 shows the results of that analysis. The same pattern of results are again obtained; indeed, the p value for the conditioning and clamp t-tests has diminished, whereas that for the pseudoconditioning procedure has increased. This supports the conclusion that the conditioning and clamp procedures produced an increase in the cell's response to the test procedure while the pseudoconditioning procedure did not. As before, the current injection procedure resulted in a highly significant decrease in the magnitude of response to the test stimulus.

Many of the subsequent analyses will be based on those cells upon which all four procedures were successfully performed. The following analysis was performed which included only such cells. The results are presented in Table 5. The same pattern of results occurred, with the conditioning and clamp procedures producing an increase in responsiveness, the current injection procedure producing a decrease, and the pseudoconditioning procedure having apparently no effect. The decreased levels of significance in Table 5 compared to Table 4 may reflect the diminished number of cells meeting the more stringent requirements.

In order to compare the effects of one procedure to another

TABLE 4

COMPARISONS OF THE MEAN POSTSYNAPTIC POTENTIAL
BEFORE AND AFTER EACH PROCEDURE
USING ONLY THE FIRST INSTANCE OF A PROCEDURE

PAIRS	N	MEAN	DEVIATION	t	df	p (2 tailed)
BEFORE CONDITIONING	92	4.92	3.58	2.25	91	0.027
AFTER CONDITIONING		5.45	3.46			
BEFORE PSEUDO-CONDITIONING	89	5.22	3.51	0.08	88	0.94
AFTER PSEUDO-CONDITIONING		5.20	3.51			
BEFORE CLAMP	93	6.80	4.52	1.80	92	0.074
AFTER CLAMP		7.25	4.57			
BEFORE CURRENT INJECTION	97	5.10	3.86	4.59	96	0.000
AFTER CURRENT INJECTION		4.50	3.47			

TABLE 5

COMPARISONS OF THE MEAN POSTSYNAPTIC POTENTIAL
BEFORE AND AFTER EACH PROCEDURE
USING ONLY CELLS WHICH ALL FOUR PROCEDURES HAVE BEEN PERFORMED UPON

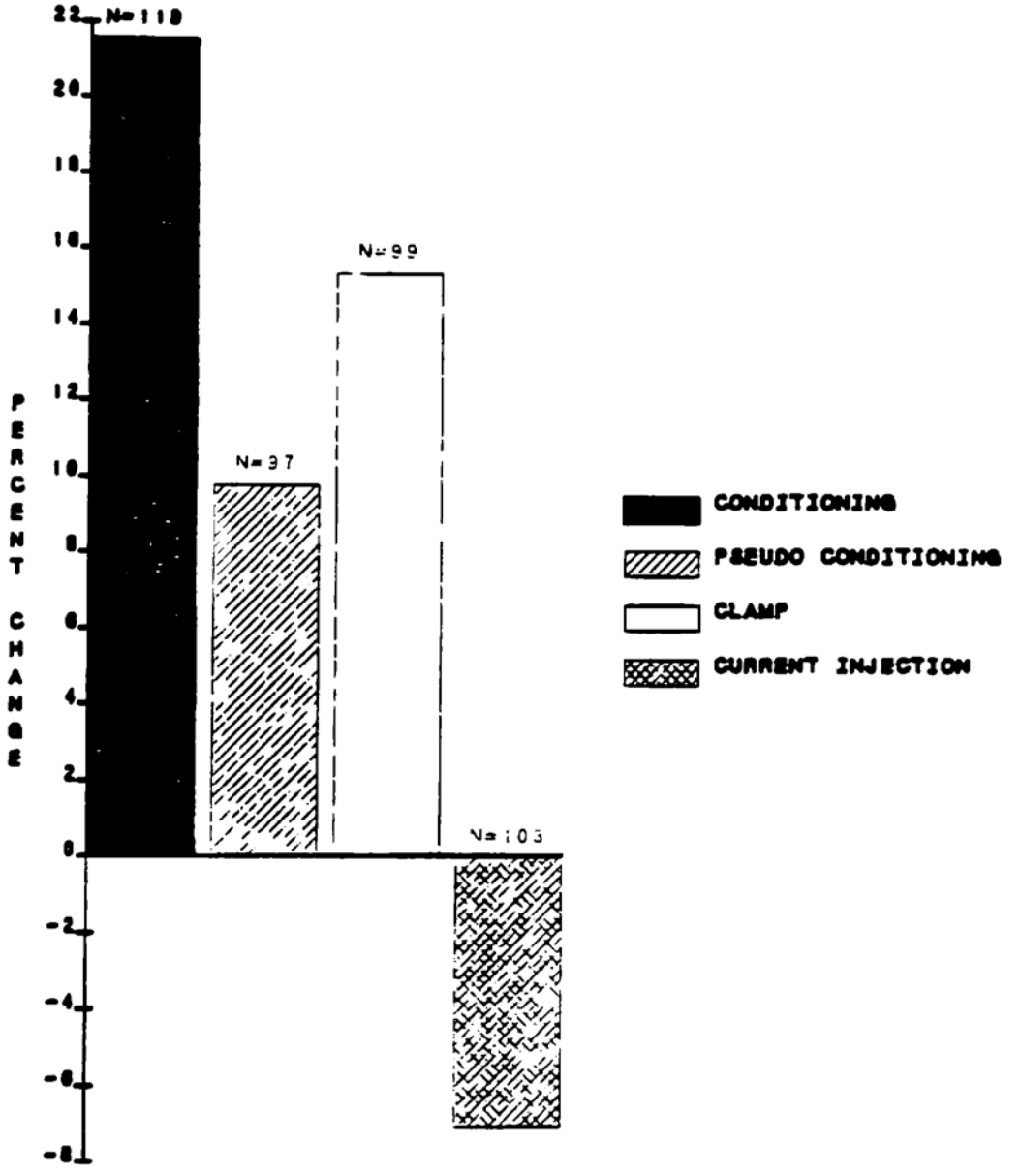
PAIRS	N	MEAN	DEVIATION	t	f	p (2 tailed)
BEFORE CONDITIONING	72	5.28	3.78	1.95	71	0.055
AFTER CONDITIONING		5.83	3.55			
BEFORE PSEUDO-CONDITIONING	72	5.34	3.63	0.17	71	0.865
AFTER PSEUDO-CONDITIONING		5.29	3.52			
BEFORE CLAMP	72	7.05	4.57	1.66	71	0.102
AFTER CLAMP		7.53	4.60			
BEFORE CURRENT INJECTION	72	5.26	4.03	3.88	71	0.000
AFTER CURRENT INJECTION		4.61	3.49			

and to eliminate cell to cell variations in the size of the PSP, the percent change in the magnitude of the PSP produced by the procedure was calculated. The percent change equals the after-procedure value of the PSP minus the before-procedure value of the PSP divided by the before-procedure value of the PSP times 100. Figure 6 shows the average percent increase or decrease in responsiveness produced by each of the four procedures. The data summarize the results of every instance of a procedure, even if repeated on the same cell.

The effects of the conditioning procedure seem to be quite similar to those obtained by Kandel and Tauc (1965a). Kandel and Tauc reported 15 of 90 (17%) of the cells they investigated showed a 40% or more increase in responsiveness after the conditioning procedure. In this study, the conditioning procedure was performed a single time upon 92 cells. Twenty cells, representing 22% of the total, showed a 40% or greater increase in responsiveness after conditioning. In addition, Kandel and Tauc reported that their 15 "conditionable" cells showed an average 122% increased responsiveness with a standard deviation of 98.0. The 20 cells of this study with a 40% or greater increase in responsiveness showed a nearly identical 121.6% mean increase and standard deviation of 64. If R2 cells are excluded from the above calculations, then 16 out of 82 unidentified cells (20%) show a greater than 40% increase with a mean increase of 130% and a standard deviation of 68.5.

Wurtz et al. (1967) report that none of 35 cells in which a

FIGURE 6
MEAN PERCENT CHANGE BY PROCEDURE



test PSP was paired with cell firing produced by current injection demonstrated an increase in amplitude of the test stimulus after pairing. Indeed, they report, "any change which did occur was a decrease in amplitude" (p. 363), although they do not report specific figures. In this experiment, the current injection procedure produced a 7.1 percent average decrease in responsiveness. This decrease, although small, appears to represent the effect of the procedure. A t-test on 103 cases comparing the magnitude of the PSP before the current injection procedure with that afterwards yields a p value less than .0001 (Table 3).

Comparisons of the Procedure Effects

Even though a given procedure may or may not have had a significant effect on the cell's response to stimulation, the effects of each procedure may or may not differ from one other. It is possible that two procedures both have a significant effect upon the cell's responsiveness, but the effects do not differ. Conversely, two procedures which may not in themselves demonstrate a significant effect, may, when compared to each other, show significant differences. Finally, one procedure which by itself causes a significant increase in responsiveness may, when compared to another which failed to show a significant change, result in an insignificant difference between the two procedures.

Comparisons of procedure effects are important for several

reasons. First, in order to be able to argue that the procedures represent a neuronal analogue of conditioning procedures, it is important that we see analogous results. That is, one might expect the conditioning procedure to produce a significant increase in responsiveness compared to the pseudoconditioning control procedure. Only in this case does it make sense to call the results of the conditioning procedure "conditioning." Second, at some point it would be useful to classify individual cells as conditionable, pseudoconditionable, or unconditionable. The ability to do so rests in large part on the demonstration of differential effects of the procedure. For example, it makes no sense to call "conditionable" those cells showing a greater than forty percent increase in responsiveness after the conditioning procedure if the conditioning and pseudoconditioning procedures produce indistinguishable effects on the cells. Finally, any analysis of the mechanism of any observed conditioning will be enhanced by demonstrable differences in the results produced by the four procedures. It would be difficult to draw conclusions about the mechanism of conditioning if all four procedures produced essentially the same effects upon the cells.

A repeated measures analysis of variance was performed in order to test for differences between group percent change means. In order to perform the analysis, only those cells upon which all four procedures had been performed could be included, and only the first instance of a procedure was included. A total of 72 cells met these conditions, and the ANOVA results are shown in Table 6. The listed p

TABLE 6

ANALYSIS OF VARIANCE COMPARING
PROCEDURE PERCENT CHANGES

SOURCE OF VARIATION	SUM OF SQUARES	df	MEAN SQUARE	F	PROBABILITY
BETWEEN CELLS	290,682	71	4,094		
BETWEEN PROCEDURES	46,457	3	15,485	7.25	.00012
RESIDUAL (ERROR)	455,140	213	2,136		
TOTAL	792,279	287	2,760		

GRAND MEAN 9.83%

value indicates the probability that the effects of all four procedures are the same. Given this low probability that the four procedures had the same effect, it is possible to look for pairwise comparisons of the effects of the procedures.

One approach to pairwise comparisons of the procedure effects is to do post-hoc contrasts. This is undesirable because this method limits the tests to those cases in which all four procedures have been performed. In order to obtain increased power, it would be more desirable to perform a test that considers every case in which the two procedures were successfully performed on a cell. Paired t-tests would accomplish this, but with four procedures, 6 tests can be performed. In order to control for an increased probability of committing a Type I error, the Bonferroni method (Wallenstien, Zucker, and Fleiss, 1980) was applied to create appropriately conservative individual alpha levels. Choosing a .05 level of significance combined with six tests leads to a p value of .008. Thus, if a p value is equal to or less than .008 in a paired t-test of the effect of any two procedures, it was deemed significant.

Table 7 shows the results of the six t-tests comparing the percent change in the magnitude of the PSP produced by the four procedures. The tests included every cell in which both procedures of interest had been performed. The varying number of cases reflects the varying distribution of the successfully performed procedures. The table lists two-tailed probabilities; however in some cases a

TABLE 7

PAIRWISE COMPARISONS OF THE PERCENT CHANGE
PRODUCED BY THE VARIOUS PROCEDURES

PAIRS	N	MEAN	DEVIATION	t	df	p (2 tailed)
CONDITIONING	86	24.35	60.41	2.52	85	0.014
PSEUDO-CONDITIONING		6.95	49.51			
CONDITIONING	83	21.91	60.97	1.00	82	0.32
CLAMP		14.29	56.72			
CONDITIONING	87	24.79	60.42	4.14	86	0.000
CURRENT INJECTION		-6.39	32.81			
PSEUDO-CONDITIONING	83	8.16	50.77	0.59	82	0.558
CLAMP		12.15	52.04			
PSEUDO-CONDITIONING	85	7.07	50.61	1.93	84	0.057
CURRENT INJECTION		-6.56	33.73			
CLAMP	89	14.15	54.62	3.46	88	0.001
CURRENT INJECTION		-9.99	28.06			

one-tailed test is appropriate. The hypothesis that the conditioning procedure leads to a greater increase in responsiveness than does the pseudoconditioning procedure is supported with a one-tailed p value of .007 which is less than the .008 cutoff. The effect of the conditioning procedure does not differ significantly from that of the clamp procedure.

Table 7 also shows that the current injection procedure has effects significantly different from those of the clamp and conditioning procedures. Specifically, it produces a significant decrease in responsiveness compared to both the conditioning and the clamp procedures. Although the p value of the test between the current injection and pseudoconditioning procedures is greater than the .008 cutoff, the difference does approach significance. This further strengthens the impression that the current injection procedure activly inhibits conditioning, rather than simply having no effect.

Effects Upon Various Cell Populations

Up to this point in the discussion, the effects of the procedures have been analyzed irrespective of the cell type upon which the procedure was performed. It is possible, however, to break down the cells into various identifiable subpopulations and to investigate the effects of the procedures upon these populations. This allows comparisons with previous work as well as further elucidation of the

process underlying conditioning.

Two methods of identifying cell populations are possible. The cell types can be functionally defined by the effects of the procedures. That is, they can be divided functionally into conditionable and unconditionable cells. Such a breakdown may provide information about the mechanism(s) of conditioning. Another way to investigate the effects of the procedures is to examine their effects upon specific, morphologically identified cells. In this experiment two identified cell populations were investigated, R2 cells and RB cells. The effects of the procedures upon these two cell types was investigated after a functional breakdown was first performed.

Effects Upon Functionally Defined Cell Types

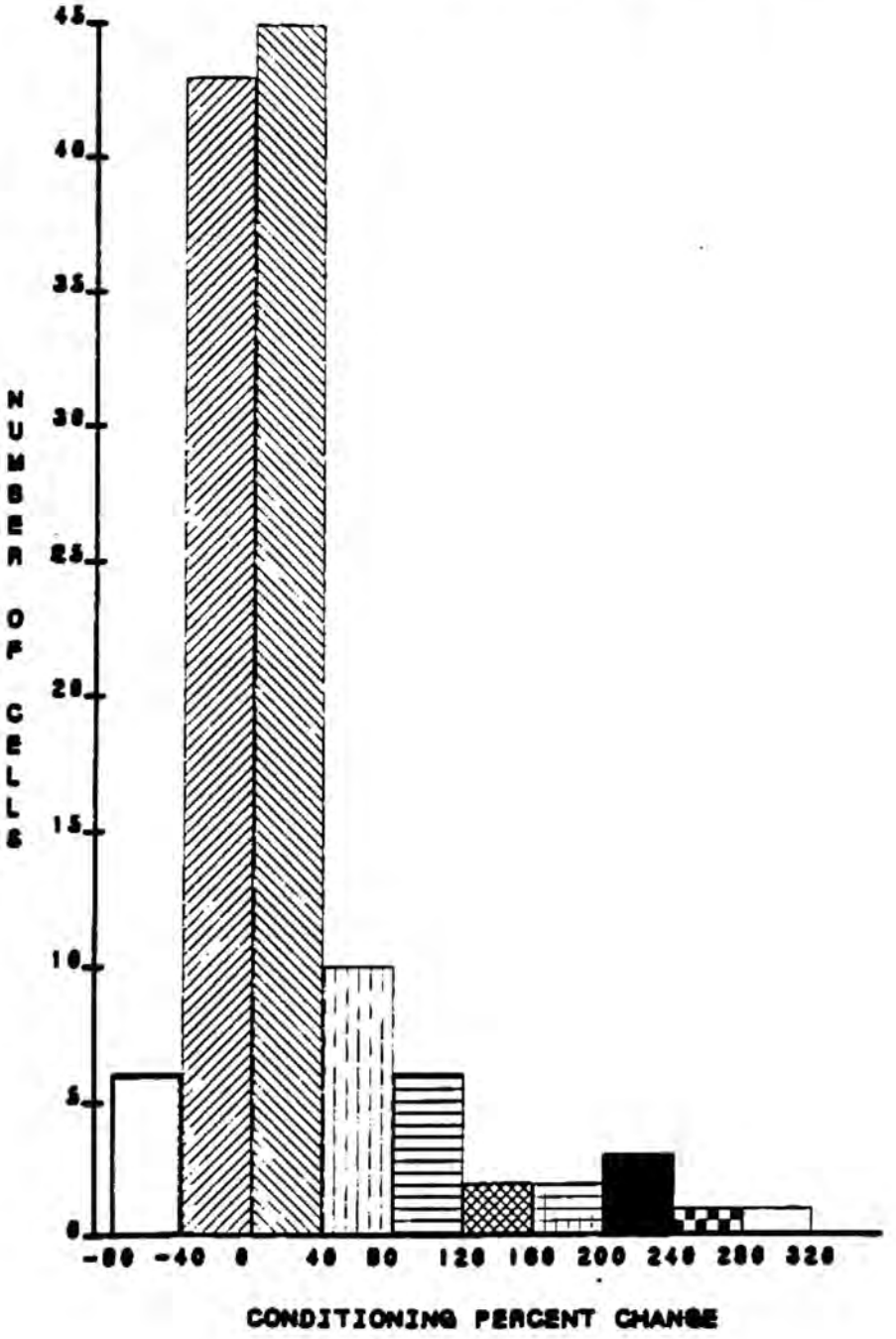
Although much can be learned by considering the effects of the procedures irrespective of cell type, there are at least two reasons for attempting to define functionally varied cell types. First, similar definitions will allow comparisons between the present results and previous results reported in the literature. Kandel and Tauc (1965a, b) define cells showing a greater than 40 percent increase in responsiveness to the conditioning procedure "conditionable". Similarly, R2 is called "pseudoconditionable." Unfortunately, Kandel and Tauc offer no clear guidelines for these definitions, and thus they are of limited usefulness.

The second reason for attempting to define cell types functionally is that it may reveal information about the mechanism(s) underlying conditioning. If distinct cell types can be identified, then it may be possible to attribute the differences to the operation of different mechanisms. Since the investigation of possible mechanisms of conditioning is one of the fundamental goals of this study, this analysis has obvious relevance.

The most desirable method of classifying cells would be one based on clear trends in the data. For example, if a plot of the percent change produced by the conditioning procedure showed a clear bimodal distribution with one group centered near 0 and another group clustered around some higher value, it would be appropriate to divide the cells into conditionable and unconditionable subpopulations. No such separation is apparent, however, in a plot of the effect of the conditioning procedure. Figure 7 is a histogram of the number of cells producing a given percent change in response to the conditioning procedure. It shows a unimodal peak centered on 0 and only the barest hint of another peak between 200 and 240 percent.

In the absence of an obvious distinction, an arbitrary one must be selected. A median split was chosen for the initial analysis since it is the most simple, obvious, and straightforward beginning. In this case, a median must be slected in order to perform the median split. There are several possibilities: the median based upon every instance of a procedure; the median of the first, independent instance

FIGURE 7
NUMBER OF CELLS RESPONDING WITH A GIVEN
CONDITIONING PERCENT CHANGE



of a procedure; or the median of a procedure in the cases where all four procedures have been performed upon a cell. Since most of the subsequent analyses will involve most of the procedures, it was deemed reasonable to select for further consideration only those cells upon which all four procedures had been performed. There were 72 such cells. For these cells, the conditioning procedure produced a mean 26.9% change with a median of 4.925%. This median was used to divide the cells into two groups of 36 cells each, called unconditionable and conditionable. In the original group of 72 cells, the pseudoconditioning procedure produced a mean 8.2% increase in responsiveness with median of .01%. Dividing the cells into two groups based on this median had the effect of separating the conditionable cells into those that responded positively and those that were inhibited by the pseudoconditioning procedure. Thus, the 36 conditionable cells were divided into one group of 23 cells which had a positive response to the pseudoconditioning procedure. These cells were called pseudoconditionable because they responded to both procedures. This left 13 cells which had a value greater than the median response to the conditioning procedure and which responded negatively to the pseudoconditioning procedure. These were called truly conditionable. The question explored was what, if any, differences exist between these cell types?

Table 8 shows 4 pairwise comparisons of the effects of the clamp and current injection procedures upon the three cell types. Since the clamp procedure is a mimic of the conditioning procedure, we

TABLE 8

COMPARISONS OF THE RESPONSE OF FUNCTIONAL CELL TYPES TO CLAMP AND CURRENT INJECTION PROCEDURES

PAIRS	N	PERCENT CHANGE CLAMP	2 TAILED P VALUE	PERCENT CHANGE CURRENT INJECTION	2 TAILED P VALUE
CONDITIONABLE	36	25.76	0.073	-8.19	0.782
UNCONDITIONABLE	36	2.67		-10.10	
UNCONDITIONABLE	36	2.67	0.913	-10.10	0.395
TRUE CONDITIONABLE	13	3.94		1.86	
UNCONDITIONABLE	36	2.67	0.047	-10.10	0.542
PSEUDO-CONDITIONABLE	23	38.08		-13.88	
PSEUDO-CONDITIONABLE	23	38.08	0.092	-13.88	0.268
TRUE CONDITIONABLE	13	3.95		1.86	

might expect that the conditionable cells would be more responsive to the clamp procedure than the unconditionable cells. The first comparison of Table 8 shows that this expectation is confirmed. The conditionable cells show a mean 25.8% increase to the clamp whereas the unconditionable cells have a mean 2.7% increase. It should be noted that there is no difference in their response to the current injection procedure. These results support the classification by cell type since the conditionable cells respond as expected to a procedure quite similar to conditioning.

The second comparison of Table 8 between the unconditionable and truly conditionable cells shows that these cell types do not differ significantly in their response to either the clamp or current injection procedures. This is somewhat unexpected because the previous result had shown a difference in the clamp procedure between the unconditionable and conditionable cells. If this difference is not due to the truly conditionable cells, does it arise from the the pseudoconditionable cells? The third comparison of Table 8 suggests that this is indeed the case. The response of the pseudoconditionable cells to the clamp procedure differs significantly from that of the unconditionable cells.

This difference between the comparisons of the pseudo- and truly conditionable cells to the unconditionable cells suggests a possible difference between the truly and pseudoconditionable cells. The fourth comparison of Table 8 directly compares the effect of the

clamp and current injection procedure on the pseudo and truly conditionable cells. It shows that the pseudoconditionable cells are substantially more responsive to the clamp procedure than are the truly conditionable cells.

Effects Upon Identified Cell Populations

Two separate identified cell populations, the RB and R2 cells, were examined in sufficient detail to justify some conclusions. Eight R2 cells and 14 RB cells were investigated. Since RB cells were somewhat difficult to identify positively on occasion, the 14 RB cells include 6 which were not as certainly identified, and these will be called "RB?." Table 9 shows the mean percent change produced in the different cell populations by the four procedures.

Most apparent from Table 9 is that the R2 cells show an apparently greater response to the conditioning, pseudoconditioning, and clamp procedures than do the RB and RB? cells. It would be interesting to compare these results to those obtained by Kandel and Tauc (1965a) on the R2 cells they investigated, but unfortunately no summary statistics were reported by Kandel and Tauc. They did report, however, that the response to the test stimulus after their conditioning procedure increased from 100 to 700 percent. This differs markedly from the present study in which the percent change ranged from -2 to 115. An explanation for this difference may lie in the difference in the priming stimulus used in the two experiments.

TABLE 9

MEAN PERCENT CHANGE PRODUCED BY THE PROCEDURES IN IDENTIFIED CELLS

CELL TYPE	N	CONDITIONING	PSEUDO-CONDITIONING	CLAMP	CURRENT INJECTION
R2	8	47.30	53.75	36.65	-13.93
RB	8	7.15	-5.77	19.47	-13.08
RB?	6	20.91	-0.45	16.89	-15.27
RB + RB?	14	13.05	-3.49	18.44	-13.90

Kandel and Tauc (1965a, b) note that the increase in responsiveness shown by a cell is directly proportional to the strength of the priming stimulus employed. In their experiments, they used the maximally effective priming stimulus. In this experiment the strength of the priming stimulus was minimized. This was done in order to facilitate the performance of the clamp procedure. In order to hyperpolarize the cell to a point at which the priming stimulus was no longer capable of firing the cell, it was frequently necessary to use a less than maximal priming stimulus. If a more powerful priming stimulus had been used, Kandel and Tauc's results suggest a greater conditioning effect would have been observed, but it would have been impossible to hyperpolarize the cell sufficiently to prevent cell firing in response to the priming stimulus.

Although Table 9 shows that the R2 cells showed similar increases in responsiveness to the conditioning and pseudoconditioning procedures, it would be useful to determine more accurately whether or not the R2 cells are pseudoconditionable. One method of investigating this question is to use the classifications based on median splits developed in the previous section. If R2 cells are pseudoconditionable, they should be well represented in the pseudoconditionable cell population defined above. This, indeed, turns out to be the case. Of the 7 R2 cells upon which all 4 procedures were performed, 6 were pseudoconditionable according to the criteria defined above. The seventh cell was unconditionable.

The RB cells show a less clear-cut picture. Table 10 shows a breakdown of the number of each cell kind in each of the three conditionability classifications. The fact that 50% of the RB cells were truly conditionable while 67% of the RB? cells were unconditionable suggests the possibility that many of the RB? cells may have been incorrectly identified as RB cells. If this is so, it raises the possibility that true RB cells are, in fact, true conditionable cells. The number of cells studied, however, is too few to reach any firm conclusion.

TABLE 10

IDENTIFIED CELLS CLASSIFIED FUNCTIONALLY

CELL TYPE (IDENTIFIED)	CELL TYPE (FUNCTIONAL) UNCONDITIONABLE	PSEUDOCONDITIONABLE	TRULY CONDITIONABLE	TOTAL
RB?	4	1	1	6
RB	2	2	4	8
RB + RB?	6	3	5	14
R2	1	6	0	7

Order Effects

An issue which could potentially cloud the interpretation of these data is the effect of the order in which the procedures were performed. In the experiments by Kandel and Tauc (1965a, b), it appears, although it is not specifically stated, that their conditioning procedure was always performed upon the cell first and then followed by any other control procedure they might have employed. Since the order was not counterbalanced, the results of the latter procedures may have been "contaminated" by the conditioning procedure. Even though the magnitude of the response to the test stimulus may have returned to baseline levels before performing a subsequent procedure, it is possible that some sequelae of the previous conditioning procedure may alter the effectiveness of the subsequent procedure. This concern is not merely academic since such effects are easily demonstrated in in vivo conditioning.

In order to control for this possibility, the four procedures in this experiment were performed at least twice in every one of the 24 possible orders. (The 24 orders are given in Appendix 2.) Since each of the 24 procedure orders was not performed only twice (some were performed three or more times), the procedure types are not exactly apportioned between the procedure orders. Table 11 shows the distribution of procedure type by procedure order. The table shows the percentage of time a given procedure type (conditioning, pseudoconditioning, etc.) was performed at the indicated procedure

TABLE 11

DISTRIBUTION OF THE PROCEDURES WITHIN SEQUENCE ORDERS

PROCEDURE TYPE	PROCEDURE ORDER							
	FIRST	SECOND	THIRD	FOURTH	FIFTH	SIXTH	SEVENTH	EIGHTH
CONDITIONING	21.0%	25.7	23.6	30.7	48.2	40.0	25	50
PSEUDO-CONDITIONING	22.5%	20.8	30.3	18.7	18.5	40.0	25	0
CLAMP	23.4%	26.7	22.5	25.3	22.2	10.0	25	0
CURRENT INJECTION	27.0%	26.6	23.6	25.3	11.1	10.0	25	50
TOTAL CASES	111	101	89	75	27	10	4	2

order. The number of cases indicates the number of cells upon which the indicated number of procedures was successfully performed. That is, a first procedure was performed upon 111 cells, a second upon 101, etc.

Under a perfect distribution, we would expect each procedure to have been performed 25% of the time in each position. For the first four procedures, this expectation was reasonably well confirmed. The fifth procedure, however, was a conditioning procedure more often than one-quarter of the time. This was because the conditioning procedure was selected to verify or rule out some previously observed outcome after performing the first four procedures as dictated by the random selection of one of the 24 procedure orders. That is, if inspection of the chart record appeared to indicate that the cell was truly conditionable, another conditioning procedure was performed to verify this impression. Since the conditioning procedure revealed the most information about a cell (whether negative or positive), it was more frequently chosen than the other procedures.

An effect of order could be due to an effect of time. With the passage of time some characteristics of the cell may change so that procedures performed later would have results different from those performed earlier. For example, the cell may (and usually does) stabilize with time after penetration. On the other hand, the cell may deteriorate. Figures 8 and 9 display two measures of the effect of the test stimulus versus the sequence of procedures performed.

FIGURE 8
MEAN PSP BEFORE AND AFTER EACH PROCEDURE NUMBER

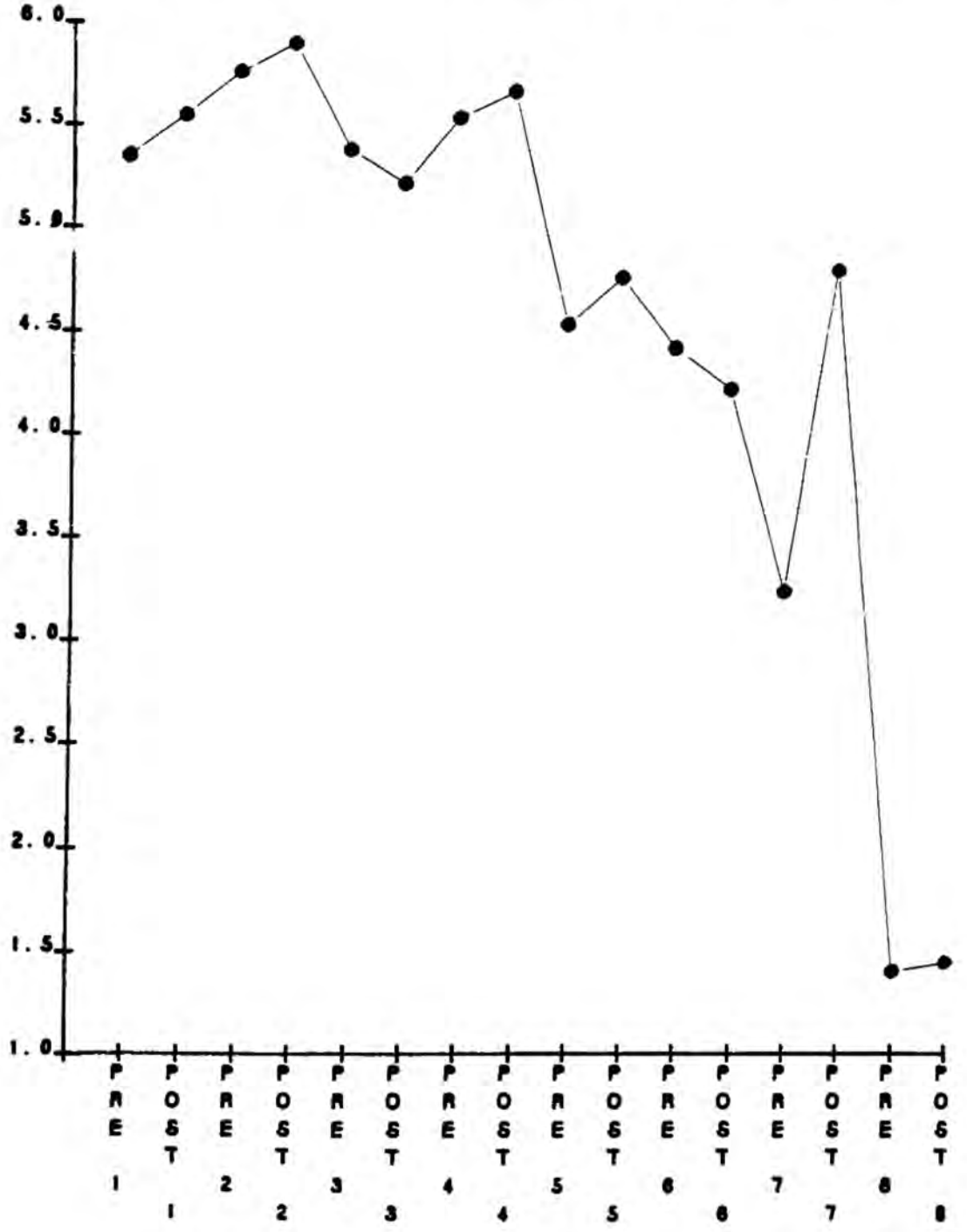


FIGURE 8
MEAN PERCENT CHANGE BY PROCEDURE NUMBER

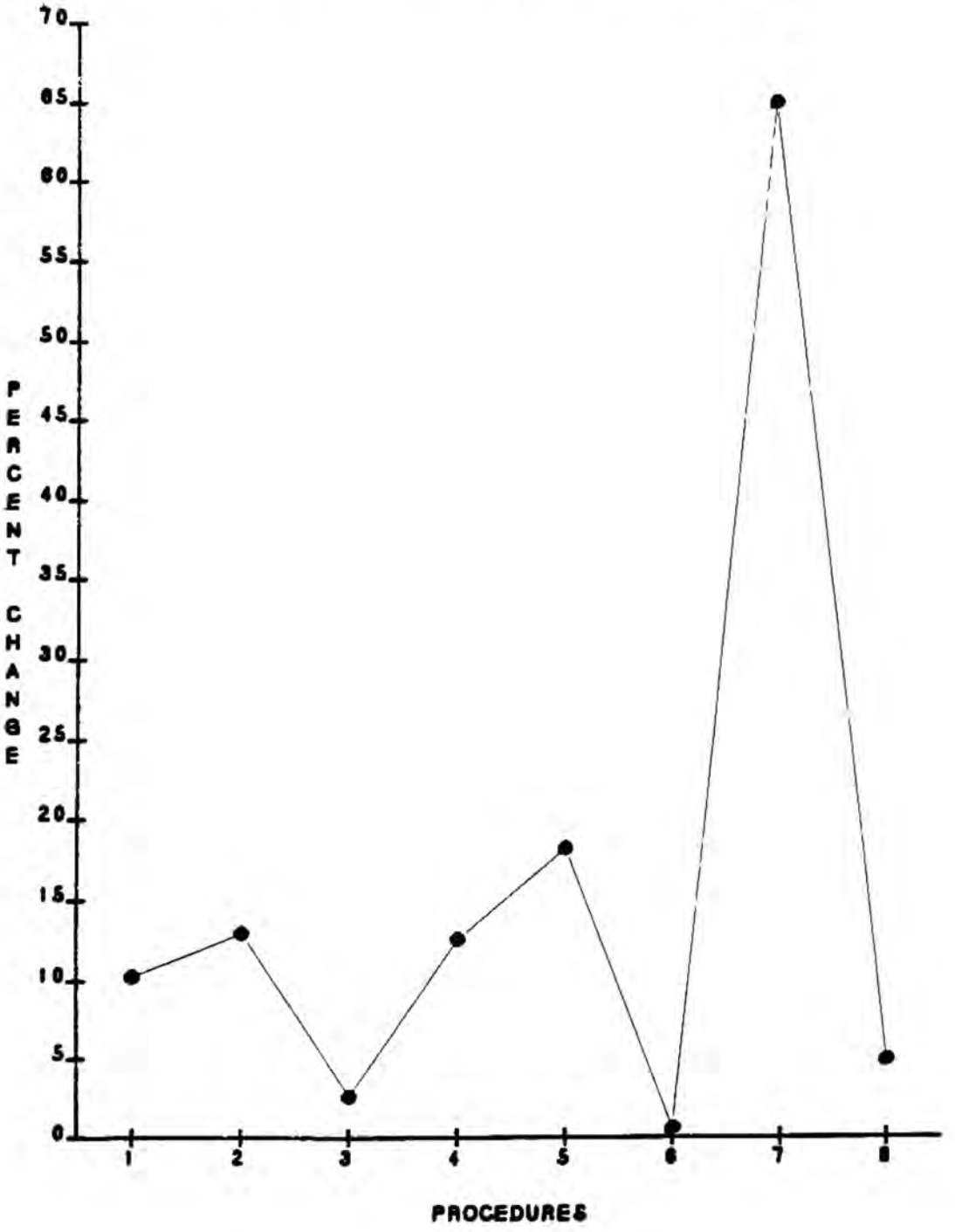


Figure 8 shows the average magnitude of the PSP produced by the test stimulus before and after each procedure number regardless of the procedure type. The apparent trailing off of effectiveness in the last several procedures may be due to deterioration; however these procedures were performed upon substantially fewer cells and the apparent decay may be an artifact. There is no apparent effect during the first four procedures. Figure 9 shows the percent change in responsiveness produced by the first, second, third, etc., procedures performed. Again, the values for the first four procedures are quite similar. The peak at the seventh procedure is almost certainly artifact produced by the fact that 7 procedures were performed upon only 4 cells.

In order to directly test for an effect of order, a second repeated measures ANOVA was performed on the procedure order. The first procedure performed on the cell, irrespective of the type, is compared to the second and the third and the fourth. The percent change in the magnitude of the response to the test stimulus was the variable compared. Table 12 gives the results of this analysis. The analysis indicates that the probability that the first four procedures (regardless of kind) had identical effects is .58. Thus there is insufficient evidence to reject the hypothesis that the first four procedures had an equal effect on the cell. A detailed summary of the effect of each procedure as a function of the ordinal position in which it was performed can be found in Appendix 4.

TABLE 12

ANALYSIS OF VARIANCE COMPARING PROCEDURE ORDER PERCENT CHANGES IRRESPECTIVE OF PROCEDURE TYPE

SOURCE OF VARIATION	SUM OF SQUARES	df	MEAN SQUARE	F	PROBABILITY
BETWEEN CELLS	304,873	73	4,176		
BETWEEN PROCEDURES	4,452	3	1,484	0.65	0.5837
RESIDUAL (ERROR)	500,010	219	2,283		
TOTAL	809,376	295	2,743		

GRAND MEAN 10.75%

The above analyses address one aspect of the question concerning the effect of the order in which the procedures were performed. It rules out an explanation of the difference between the conditioning and pseudoconditioning effects as being due, for example, to the conditioning procedure being performed first on a dying cell. Under those circumstances it would come as no surprise that the conditioning procedure was more effective than the subsequent pseudoconditioning procedure. It does not rule out, however, the possibility that a specific order may have had effects different from another order. Further, it is still possible that a specific procedure may affect a subsequent procedure. For example, the potentially destructive effects of excessive hyperpolarization during the clamp procedure might affect the outcome of subsequent procedures. This apparently simple suggestion contains a plethora of testable hypotheses. For instance, does the clamp adversely affect the next procedure, regardless of kind, or only a specific procedure? If it affects a specific procedure, which one? This experiment, however, was not designed to answer such questions; therefore, with one exception, they will not be considered further.

Conditioned Inhibition

Animal conditioning experiments have shown that the history of an organism's experience plays an important role in the process of conditioning. If a potential CS has been experienced by an organism before it is paired with the UCS, the acquisition of a conditioned

response can be strongly affected. Specifically, if the CS has been presented unpaired with a UCS, acquisition of a conditioned response is delayed in a process called conditioned inhibition. In this experiment during the pseudoconditioning procedure the priming stimulus is presented midway between two test stimuli. Thus, the test stimulus could come to signal a 10-second period during which the priming stimulus will not occur. In in vivo conditioning we would expect such a procedure to result in conditioned inhibition. When the CS is now paired with a UCS, the development of a subsequent conditioned response would proceed more slowly. Does a similar phenomenon occur at the neuronal level in this analogue of conditioning?

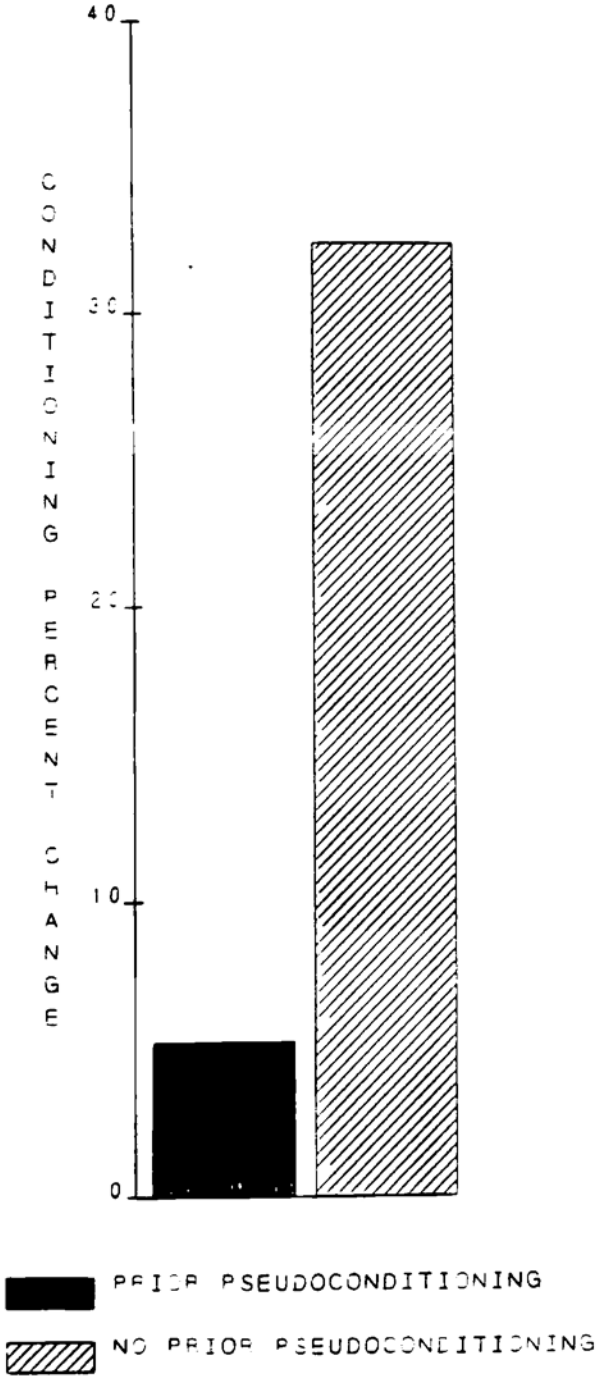
The importance of this question is twofold. First, if such a conditioned inhibition is occurring, it affects our interpretation of the data. In approximately 25 percent of the cases, the pseudoconditioning procedure is performed just before the conditioning procedure. If the pseudoconditioning procedure results in the development of conditioned inhibition, we would expect the subsequent conditioning procedure to be less effective. That is, we would expect a lesser increase in the effectiveness of the test stimulus to develop. If so, then our estimate of the effectiveness of the conditioning procedure would be negatively biased. The conditioning procedure would be more effective than it appears. Second, if there is an inhibitory effect of the pseudoconditioning procedure, then it would support the proposition that the neuronal model this experiment

has explored is a meaningful analogue of *in vivo* conditioning. For these two reasons it is worthwhile to examine this particular order effect.

In order to discover a possible effect of the pseudoconditioning procedure on the conditioning procedure, the analysis was conducted as follows: Cases were selected for further analysis if both the pseudoconditioning and the conditioning procedures had been performed at least once upon a cell. The cases were then divided into two groups. One group consisted of the cases in which the pseudoconditioning procedure immediately preceded the conditioning procedure. The other group consisted of all the other cases. This included instances where the conditioning procedure was performed first and when it was separated from the pseudoconditioning procedure by at least one other procedure. In 24 of the 82 cases in which both procedures were performed, the pseudoconditioning procedure immediately preceded the conditioning procedure.

Figure 10 shows the percent increase in the response to the test stimulus after the conditioning procedure in those cases in which the pseudoconditioning procedure preceded the conditioning procedure and those in which it did not. Prior exposure to pseudoconditioning produced a significant decrease in the response to the conditioning procedure ($t=2.17$, $df=64.82$, two-tailed $p=.034$, in a separate variance estimate).

FIGURE 10
THE EFFECT OF PRIOR PSEUDOCONDITIONING
ON CONDITIONING



DISCUSSION

In many ways these results replicate previous work. The effects of the procedures employed here closely parallel previously reported results. The conditioning procedure was almost identical to that reported by Kandel and Tauc (1965a) and the results were strikingly similar. The reported percentage of cells showing a 40% or greater increase in responsiveness was almost identical. In addition, both the mean increase and the variation in responsiveness for these cells was the same in the two studies. The similarity of these results suggests that the conditioning procedures employed in the two studies are comparable and that the effects of the procedure are fairly generalizable, at least to cells in the abdominal ganglion of the Aplysia.

The effects of the current injection procedure parallel the results of Wurtz et al. (1967). Both studies show that in the general cell population a postsynaptic potential paired with postsynaptic cell firing per se does not lead to an enhanced PSP but rather, as this study showed, such pairings produce an unequivocal decrease in the magnitude of the PSP. Further, it showed that this result can be extended to pseudoconditionable cells as well. Indeed, the current injection procedure produced a decreased PSP in all but one cell group studied, no matter how they were classified. Only truly conditionable cells showed an increase in the magnitude of the PSP after the current injection procedure. This increase was not large enough, however, to

differ significantly from the decreases produced in other cell types.

The results of the clamp procedure were also quite similar to the previous work. Although Kandel and Tauc (1965b) only applied the clamp procedure to R2 cells, they reported that it, like the conditioning procedure, led to an enhanced responsiveness. This study's results replicated that finding and also showed that this was true of cells in general as well as pseudoconditionable cells other than R2. Generally, then, the effects of the clamp procedure closely paralleled the effects of the conditioning procedure in that a cell responsive to the conditioning procedure was also responsive to the clamp procedure. The important exception to this was the response of the truly conditionable cells. They were responsive to the conditioning procedure but not to the clamp procedure. This may be another bit of evidence suggesting that the mechanism of true conditioning is distinct from that of pseudoconditioning.

Another area in which these results replicate previous work is the effects on R2. Similar to Kandel and Tauc (1965a, b), this experiment found R2 to be pseudoconditionable. Six of the 7 R2 cells were classified as pseudoconditionable based on median splits. As Table 9 showed, the R2 cells were highly responsive to the conditioning, pseudoconditioning and clamp procedures and unresponsive to the current injection procedure. This precisely parallels the results reported by Kandel and Tauc (1965b) in their investigation of the mechanism of the conditioning of R2 cells. There was a difference

in the extent of conditioning shown by R2 cells in the two experiments. This difference is probably the result of the difference in the strengths of the priming stimuli used in the two experiments.

Order Effects

A new finding of this study was the powerful effect of the pseudoconditioning procedure upon a subsequent conditioning procedure. The pseudoconditioning procedure not only failed to produce a significant increase in the magnitude of the response to the test PSP but also it had inhibitory effects which persisted to the point of influencing the subsequent conditioning procedure. Thus, it appears that the pseudoconditioning procedure resulted in a conditioned inhibition quite similar to that observed in conditioning experiments on whole organisms.

This result has implications both for this study and other studies. It certainly suggests the possiblity that the results of this study may underestimate the effects of the conditioning procedure. On 24 of the 82 occasions both procedures were performed on a cell, the pseudoconditioning procedure immediately preceded the conditioning procedure. The inhibition produced by the pseudoconditioning procedure diminished the observed effect of the conditioning procedure. This resulted in a skewed classification of cells such that some conditionable cells were classified as unconditionable. How this may have affected the analysis of the

effects of the clamp and current injection procedures upon the functional cell types is unknown.

This study's results have an even more interesting implication con cerning the neuronal substrates of conditioning. Conditioned inhibition is generally thought to be a "higher" process, one which requires more complex processing than simple CS-UCS conditioning because it involves inhibition rather than excitation of behavior and because it can occur over longer ISIs than conditioning. This experiment may have provided the first evidence of conditioned inhibition occurring in a simple neuronal network. If subsequent research confirms these findings, it would support a developing view of the neuron as a complex information processing unit (Klopf, 1980).

The Mechanism of Conditioning

This experiment was based on the assumption that neuronal processes underlie the changes observed in <u>in vivo</u> conditioning experiments. Specifically, the experiment sought to answer the question of whether or not electrophysiological procedures which were analogous to the stimuli sequences in whole animal conditioning would produce analogous results. If so, could these results reveal anything about the neuronal mechanism which might underlie conditioning?

In several respects the results of the electrophysiological procedures parallel the results of animal conditioning experiments:

1. The conditioning procedure, like pairing a CS and UCS with a short ISI, generally results in an increased responsiveness to the test (CS) stimulus.

2. The pseudoconditioning procedure, like pairing the CS and UCS with a long ISI, does not result in increased responsiveness. The fact that the conditioning procedure produces a significantly greater increase in responsiveness than does the pseudoconditioning procedure indicates that the effect of the conditioning procedure is apparently due specifically to the pairing of the test and priming stimuli; this is similar to _in vivo_ conditioning in which the CS must be temporally paired with the UCS for conditioning to occur.

3. The clamp procedure, identical to the conditioning procedure as far as the presentation of stimuli is concerned, produced roughly similar increases in responsiveness. Although no direct analogue of this procedure exists in _in vivo_ conditioning, the order of presentation of stimuli is identical to short ISI conditioning and similar results are obtained.

4. In the current injection procedure no powerful stimulus external to the cell, like the UCS, is presented, and no conditioning occurs. This would be the expected result if only the CS were presented to an organism.

5. When the pseudoconditioning procedure precedes the conditioning procedure, it significantly diminishes the effectiveness of the conditioning procedure. In whole animal conditioning the long ISIs of the pseudoconditioning procedure would come to signal the non-occurrence of the UCS, and subsequent conditioning would be more difficult.

The combination of the above results supports the conclusion that the electrophysiological procedures employed in this study are analogous to those which occur in the organism during conditioning. Possibly, the effect of the UCS is to produce an input like the priming stimulus at some conditionable cell while the CS produces an initially weak input like the test stimulus. The pairing of these two inputs may produce some change in the cell (or synapse) such that subsequent presentations of the CS produce an input to the cell large enough to cause it to fire and to generate the conditioned response.

If the procedures used in this study are analogous to the neuronal events which occur during in vivo conditioning, what do the experimental results reveal about the mechanism underlying conditioning? The fact that the precise pathway from either connective to an individual cell was generally unknown precludes definitive statements about the course of events underlying conditioning. The results did provide, however, some information on this question.

A major concern this investigation sought to address was whether or not postsynaptic cell firing played a part in conditioning. In this study the current injection procedure never once lead to a significant increase in responsiveness. Indeed, like the report of Wurtz et al. (1967), the current injection procedure resulted in a decrease in responsiveness. Further, when postsynaptic cell firing was prevented during the clamp procedure, pairing the test and priming stimuli still resulted in a significant increase in responsiveness (Table 4) which was not significantly different from the increase produced by the conditioning procedure (Table 7). In view of these results, a hypothesized Hebbian mechanism based on paired presynaptic and postsynaptic cell firings again fails to be supported. If pairing a test PSP with cell firing induced by the injection of depolarizing current is a reasonable operationalization of Hebb's proposal that one cell participated in the firing of another, then these results fail to support Hebb's hypothesis. If postsynaptic cell firing does play a role in conditioning, it must do so by some mechanism more complicated than simple pairing with incident PSPs.

The results did not allow a discrimination between presynaptic facilitation and the modified Hebbian model. If the enhanced response shown by the truly conditionable cells had been significant, it would have been difficult to explain in terms of presynaptic facilitation. The fact that no cell type showed a response to the current injection procedure means there is no basis for discriminating between the two mechanisms. A test to distinguish unequivocally between presynaptic

facilitation and a modified Hebbian model would require a technically extremely difficult experiment involving the simultaneous recording from several neurons which known patterns of interconnection.

Another interesting implication of this experiment is that the mechanism of conditioning may be clearly different from the mechanism of pseudoconditioning. The results reported in Table 8 indicate that the pseudoconditionable cells are uniquely responsive to the clamp procedure. There is no a priori reason to expect the truly and pseudoconditionable cells to differ in their response to the clamp procedure. Indeed, if any difference were expected, it would be that the truly conditionable cells should have been most responsive to the clamp procedure since it is quite similar to the conditioning procedure. This was not the case, however, for it was the pseudoconditionable cells which were responsive to the clamp procedure while the truly conditionable cells hardly differed from the unconditionable cells.

The responsiveness of the pseudoconditionable cells to the clamp procedure suggests that a particular mechanism of pseudoconditioning may exist. A possible mechanism is suggested by the fact that the three procedures to which the pseudoconditionable cells are responsive (conditioning, pseudoconditioning, and clamp) involve the application of the priming stimulus. This suggests that the pseudoconditionable cells are "conditionable" because of an effect of the priming stimulus. That is, the application to these cells of

the priming stimulus alone appears to produce an increase in excitability. As a result, whether or not paired with the test stimulus, or whether or not the cell is hyperpolarized, the effect of the application of the priming stimulus is to increase the cell's responsiveness to stimuli.

There are two additional items of evidence which support this hypothesis. First, a cell that appeared "conditionable" because it showed a marked and readily apparent response to the conditioning procedure frequently turned out to respond just as strongly to the pseudoconditioning and clamp procedures. In such cells the magnitude of the spontaneously occurring postsynaptic potentials also showed a marked increase after the application of the priming stimulus, whatever the procedure. This suggested that the priming stimulus produced a generalized increase in responsiveness to all inputs to the cell. Second, on occasion this hypothesis was directly tested by conducting an additional procedure, a "pseudoclamp." The cell was hyperpolarized to prevent firing, and the priming stimulus was applied with a 10 second ISI, as in the pseudoconditioning procedure. In these cells facilitation still occurred even though the cell was prevented from firing and the priming stimulus was not paired with the test stimulus. This strongly suggests that the cells are pseudoconditionable simply as a result of an effect of the priming stimulus itself.

Such a conclusion suggests that the mechanism of

pseudoconditioning may be completely different from the mechanism of true conditioning. It also implies that generalizing results obtained on pseudoconditionable cells to mechanisms of true conditioning may be inappropriate. Thus, Kandel and Tauc's (1965b) results concerning the mechanism of the pseudoconditioning observed in R2 cells may apply only to other pseudoconditionable cells and not to the processes underlying in vivo conditioning.

Identifying True Conditioning

The most apparent and disappointing weakness of the results is that they provide no clear and convincing evidence of true conditioning. The median splits performed above guarantee that at least some cells will be called "truly conditionable." One would like some parameter which clearly differentiates these cells, yet there is nothing compellingly different about the cell population as it was defined. Although this cell population was the only group that showed a positive response to the current injection procedure, the response was still not significantly different from the responses of the other cell kinds (Table 8). Although the truly conditionable cells differed from the pseudoconditionable cells in their response to the clamp procedure, this difference seems to characterize and to define the pseudoconditionable cells rather than to delineate the truly conditionable cells.

The RB cells, when clearly identified as such, seemed to be

truly conditionable. The small number of these cells plus the questionable identification of some of them preclude a convincing conclusion. If conditionable cells exist (whether truly or pseudoconditionable), a histogram of the cells' responses to the conditioning procedure should show more than just a hint of a bimodal distribution indicating conditionable and unconditionable sub-populations. The data from this investigation, however, do not allow this conclusion (Figure 7).

It should be noted that while the data do not define a truly conditionable cell population, they do support the possibility of the existence of such a group. The conditioning procedure does produce a significant increase in the response of the cell. It also produces a significantly greater increase in responsiveness than does the pseudoconditioning procedure. This implies that at least some of the effects of the conditioning procedure are specific to the pairing of the test stimulus and priming stimulus; some true conditioning must be occurring. Moreover, the inhibitory effects of the pseudoconditioning procedure blurs the effect of the conditioning procedure. Given these facts, it seems relevant to ask how truly conditionable cells might be identified.

There are several methodological changes which could be made which would improve the experimental design and facilitate the observation of true conditioning. First, the pseudoconditioning control procedure could be greatly improved. Instead of using a

constant 10 second ISI, the priming stimulus should be presented randomly within the 20 second period between one test stimulus and the next. This would have the effect of precluding the possible development of conditioned inhibition.

Second, even with the pseudoconditioning control procedure modified as suggested above, it should not immediately precede the conditioning procedure. Some inhibitory effect may still develop and diminish the effectiveness of the conditioning procedure. The importance of clearly establishing conditioning outweighs precluding an effect of order.

Third, the duration of pairing should be increased. Ten trials would not be expected to result in the development of a conditioned response in most whole animal conditioning paradigms. Frequently hundreds of trials are needed. Although Kandel and Tauc (1965a, b) reported substantial conditioning with so few trials, the results of this experiment seem to suggest that additional pairings would be advisable.

Fourth, the strength of the priming stimulus should have been increased. The requirements of the clamp procedure and the conditioning procedure were antagonistic. In order to produce conditioning the priming stimulus in the conditioning procedure should have been as strong as possible. In order to prevent cell firing during the clamp procedure, however, the priming stimulus had to be

reduced. In order to simplify interpretation, only the lesser strength stimulus was used. The cost, however, may have outweighed the benefit.

Finally, the experiment should be performed upon cells with known monosynaptic input pathways for both the test and priming stimuli. This would permit much more precise interpretations of the results. Such an approach has recently been adopted by Kandel and his collaborators, and it has demonstrated synapses which show an increased responsiveness only when presynaptic activity is paired with a potent UCS (Carew, Hawkins and Kandel, 1983). The mechanism of these changes, however, has not yet been fully determined, and further investigation is needed in this area.

CONCLUSIONS

The neuronal basis of learning is unknown. Hebb's theory has inspired many theoretical accounts of learning, but its veracity has never been unequivocally proven by experiment.

This investigation examined the effect of various forms of stimulation of neural tissue on the conditionability of neurons in the abdominal ganglion of the Aplysia.

Pairing a weak postsynaptic potential with strong stimulation of a neuron's inputs produced a strengthening of the postsynaptic potential. This was true even when the cell did not fire in response to the strong stimulus because it had been hyperpolarized. It was not true if cell firing induced by current injection was substituted for the strong stimulation. This suggested that postsynaptic cell firing was neither necessary or sufficient to produce conditioning. The facilitation produced by pairing of the stimuli occurred only when the time interval between the stimuli was similar to the ISI which has been found to be effective in whole animal conditioning.

Classification of cells into types depending on their response to the procedures indicated that in some cells strong stimulation produced a non-specific strengthening of both induced and spontaneous postsynaptic potentials. The fact that across all cells the conditioning procedure led to a significantly greater increase in

responsiveness than did the pseudoconditioning procedure implies that not all of the effect of the conditioning procedure is due to a general increase in responsiveness caused by the priming stimulus. Some of the facilitation appears to be related to the time interval between the test and primng stimuli.

Prior pseudoconditioning diminished a cell's response to an immediately following conditioning procedure. This suggests that the history of a cell's exposure to stimulation affects its subsequent response.

The electrophysiological procedures produced neuronal results which appeared to be directly analogous to behavioral results produced by *in vivo* conditioning experiments. Although postsynaptic cell firing *per se* does not appear to produce these changes, their mechanism remains unclear.

APPENDIX 1

Summary Description of the Aplysia.

The Aplysia is a marine gastropod mollusck found throughout the oceans of the world. It is a docile herbivore which feeds on seaweed. The Aplysia begins its life cycle as an egg, one of a million such eggs deposited by a mature Aplysia. The egg hatches in one day to begin a 34 day phase as a Veliger, a free swimming larvae. It then enters a metamorphic stage where it progressively takes on the appearance of the adult animal. It reaches reproductive maturity after approximately 120 days. Reproductively, it is a hermaphrodite, capable of performing male and female roles simultaneously. When the adult is fertilized, it lays an egg mass and then dies shortly thereafter.

The Aplysia has many characteristics which make it suitable for neurophysiological investigation. The animals are easily maintained in artificial sea water aquariums. They can be bred in captivity with a generation time of 19 weeks. This makes possible the development of genetically homogeneous strains with reduced interindividual variation in physiology and behavior. There are 35 naturally occurring species of Aplysia. Although substantial differences in appearance characterize the species, their nervous systems appear to be quite similar across species. Aplysia californica inhabits the coastline of California. Aplysia depilans is

one of three common European species.

The characteristic of Aplysia which makes it most suitable for neurophysiological investigation is the animal's nervous system. The nervous system consist of only approximately 40,000 neurons located in 9 discrete ganaglia. Eight of the ganglia are located in the head in a ring which consists of 4 paired ganglia, the left and right cerebal, buccal, pleural, and pedal ganglia. The ninth ganglia, the abdominal, is located in the midst of the animal's visceral mass. The neurons of the ganglia communicate with one another and with effector organs via connectives composed of numerous nerve fibers surrounded by a protective sheath. Each of the ganglia tend to control distinct functions. The buccal ganglia, for example, consist of motor neurons which control the activity of the buccal mass, the Aplysia's chewing organ. The cerebral ganglia innervate the eyes and tentacles, thus providing sensory input. The abdominal ganglion controls visceral functions such as circulation, respiration and reproduction.

Extensive investigation has shown that the neurons of Aplysia are largely invariant across individuals. The same identifiable neurons control the same function in each animal. Individual neurons can be identified by their location in the ganglion, their size, pigmentation, and firing pattern. This fact has given rise to a system to identify individual neurons. Neurons of the abdominal ganglion are labeled with an R or L indicating their position in the right or left side of the ganglion in a dorsal view. Numbers and/or

other letters are added to indicate particular neurons. R2, for example, is the largest neuron in the animal and is located on the right side of the abdominal ganglion. It has an orange pigmentation and a diameter of up to one millimeter making visual identification possible with the naked eye even in a ganglion with the protective sheath still intact. Other prominent cells include R14 and R15, L1 through L4, L7, and L11. Figure 3 of the text shows the locations of these and other identified neurons.

The Aplysia's large, hardy neurons can be simultaneuosly penetrated by numerous microelectrodes. This has made possible the detailed study of their functioning and the interconnections between neurons. As a result, the study of this simple nervous system has enabled neuroscientists to obtain a rather complete picture of the neuronal circuitry subserving numerous overt behaviors.

APPENDIX 2

Formula for Artifical Sea Water

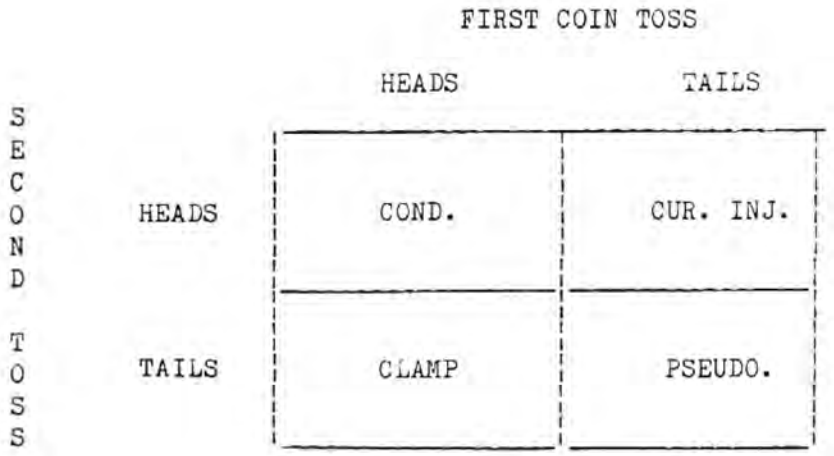
SALT	CONCENTRATION
NaCl	480 millimolar
KCl	10
$CaCl_2$	10
$MgCl_2$	20
$MgSO_4$	30
$NaHCO_3$	2

APPENDIX 3

PROCEDURE

Order	First	Second	Third	Fourth
1	Cond.	Cur.inj.	Clamp	Pseudo.
2	Cond.	Cur.inj.	Pseudo.	Clamp
3	Cond.	Clamp	Cur.inj.	Pseudo.
4	Cond.	Clamp	Pseudo.	Cur.inj.
5	Cond.	Pseudo.	Clamp	Cur.inj.
6	Cond.	Pseudo.	Cur.inj.	Clamp
7	Cur.inj.	Cond.	Pseudo.	Clamp
8	Cur.inj.	Cond.	Clamp	Pseudo.
9	Cur.inj.	Clamp	Pseudo.	Cond.
10	Cur.inj.	Clamp	Cond.	Pseudo.
11	Cur.inj.	Pseudo.	Clamp	Cond.
12	Cur.inj.	Pseudo.	Cond.	Clamp
13	Clamp	Pseudo.	Cur.inj.	Cond.
14	Clamp	Pseudo.	Cond.	Cur.inj.
15	Clamp	Cond.	Pseudo.	Cur.inj.
16	Clamp	Cond.	Cur.inj.	Pseudo.
17	Clamp	Cur.inj.	Cond.	Pseudo.
18	Clamp	Cur.inj.	Pseudo.	Cond.
19	Pseudo.	Cond.	Clamp	Cur.inj.
20	Pseudo.	Cond.	Cur.inj.	Clamp
21	Pseudo.	Cur.inj.	Clamp	Cond.
22	Pseudo.	Cur.inj.	Cond.	Clamp
23	Pseudo.	Clamp	Cur.inj.	Cond.
24	Pseudo.	Clamp	Cond.	Cur.inj.

PROCEDURE CHOICE ALGORITHM



APPENDIX 4

MEAN OF THE PERCENT CHANGES OF INDIVIDUAL CELLS AND MEAN POSTSYNAPTIC POTENTIALS AS A FUNCTION OF PROCEDURE TYPE AND SEQUENTIAL POSITION

	SEQUENTIAL POSITION OF PROCEDURE			
	FIRST	SECOND	THIRD	FOURTH
NUMBER	30	26	21	23
BEFORE COND.	4.32	5.97	5.13	3.94
AFTER COND.	4.80	6.35	5.70	4.47
% CHANGE	22.68	18.59	21.40	26.96
NUMBER	25	21	27	14
BEFORE PSEUDOCOND.	4.92	5.24	5.56	4.23
AFTER PSEUDOCOND.	5.32	5.88	4.60	4.43
% CHANGE	13.41	21.10	-11.76	9.92
NUMBER	26	27	20	19
BEFORE CLAMP	7.00	7.04	5.73	8.73
AFTER CLAMP	7.78	7.17	6.47	8.99
% CHANGE	17.02	27.71	13.35	10.62
NUMBER	30	27	21	19
BEFORE CUR. INJ.	5.32	4.71	5.12	5.28
AFTER CUR. INJ.	4.55	4.29	4.40	4.81
% CHANGE	-10.71	-12.90	-8.02	-1.65

Note: The percent change is not calculated from the means before and after each procedure. Instead the percent change values are means of the individual cells' percent changes: A cell with a low baseline value has a greater percent change for a given increase in potential than does a cell with a higher base line value. For example, a cell with a pre-procedure PSP of 1.0 and a post-procedure PSP of 2.0 has a percent change of 100%; a cell with a pre-procedure PSP of 8.0 and a post-procedure PSP of 7.0 has a percent change of -12.5%. The mean percent change is 43.8%, yet both the pre- and post-procedure PSP means are identically 4.5.

BIBLIOGRAPHY

Arbib, M. A., Kilmer, W.L. and Spinelli, D.N. (1976). Neural models and memory. In M.R. Rosenzweig and E.L. Bennet (Eds.), Neural Mechanisms and Memory. Cambridge, Mass.: MIT Press.

Bienstock, E.L., Cooper, L.N. and Munro, P.W. (1982). Theory for the development of neuron selectivity: orientation specificity and binocular interaction in visual-cortex. Journal of Neuroscience, 2, 32-48.

Bliss, T.V.P. and Lomo, T. (1973). Long-lasting potentiation of synaptic transmission in the dentate area of the anaesthetized rabbit following stimulation of the perforant path. Journal of Physiology, 232, 331-356.

Bullock, T.H. (1977). Introduction to Nervous Systems. San Francisco: Freeman.

Carew, T.J., Hawkins, R.D., and Kandel, E.R. (1983). Differential conditioning of a defensive withdrawal reflex in Aplysia californica. Science, 219, 397-400.

Carol, M.P. (1976). Neuronal circuits involved in learning: the use of modifiable synapses in the simulation of behavior. International Journal of Neuroscience, 7, 9-18.

Douglas, R.M. (1978a). The conditional nature of synaptic modification in the fascia dentata of the rat. Unpublished doctoral dissertation, Dalhousie University.

Douglas, R. M. (1978b). Heterosynaptic control over synaptic modification in the dentate gyrus. Eighth Annual Meeting, Society for Neuroscience, St. Louis, Mo.

Goddard, G.V. (1980). Component properties of the memory machine: Hebb revisited. In Jusczyk, P.W. and Klein, R.M. (Eds.) The Nature of Thought: Essays in Honor of D. O. Hebb. Hillsdale, New Jersey: Lawrence Erlbaum Associates.

Hebb, D.O. (1949). Organization of Behavior. New York: John Wiley & Sons.

Hilgard, E. R. and Bower, G. (1966). Theories of Learning. New York: Appleton-Century-Crofts.

Hubel, D. H., and Wiesel, T. N. (1962). Receptive fields, binocular interaction and functional architecture in the cat's visual cortex. Journal of Physiology, 160, 106-154.

Kandel, E.R. (1976). Cellular Basis of Behavior. San Francisco: W.H. Freeman and Co.

Kandel, E.R. (1979, September). Small systems of neurons. Scientific American, 241, No. 3, 66-87.

Kandel, E.R. and Tauc, L. (1965a). Heterosynaptic facilitation in neurons of the abdominal ganglion of Aplysia depilans. Journal of Physiology, 181, 1-27.

Kandel, E.R. and Tauc, L. (1965b). Mechanisms of heterosynaptic facilitation in the giant cell of the abdominal ganglion of Aplysia depilans. Journal of Physiology, 181, 28-47.

Kandel, E.R. and Schwartz, J.H. (1982). Molecular biology of learning: modulation of transmitter release. Science, 218, 433-443.

Kandel, E.R. and Spencer, W.A. (1968). Cellular neurophysiology and learning. Physiological Review, 68, 65-114.

Klopf, H.A. (1982). The Hedonistic Neuron: A Theory of Memory, Learning, and Intelligence. Washington, D.C.: Hemisphere Publications.

Little, W.A. and Shaw, G.L. (1975). A statistical theory of short and long term memory. Behavioral Biology, 14, 115-130.

McNaughton, B.L. (1978). The dynamics of synaptic modulation in the medial and lateral components of the perforant pathway to the fascia dentata in the rat. Unpublished doctoral dissertation, Dalhousie University.

Pavlov, I. (1927). Conditioned reflexes. Oxford: Oxford University Press.

Rescorla, R.A., and Wagner, A.R. (1972). A theory of Pavlovian conditioning: Variations in the effectiveness of reinforcement and non-reinforcement. In A.H. Black, and W. F. Prokasy (Eds.), Classical conditioning II: Current research and theory. New York: Appleton-Century-Crofts.

Schwartz, B. (1978). Psychology of Learning and Behavior. New York: W.W. Norton and Company Inc.

Skinner, B. F. (1938). The Behavior of Organisms. New York: Appleton-Century-Crofts.

Skinner, B. F. (1950). Are theories of learning necessary? Psychological Review, 57, 193-216.

Sutton, R. S. and Barto, A.G. (1981). Towards a modern theory of adaptive networks: expectation and prediction. Psychological Review, 88, 135-170.

Takeuchi, A. and Amari, S. (1979). Formation of topographic maps and

columnar microstructures in nerve fields. Biological Cybernetics, 35, 63-72.

Thompson, R.F. (1976). The search for the engram. American Psychologist, 31, 209-227.

Wallenstein, S., Zucker, C. and Fleiss, J. (1980). Some statistical methods useful in cirulation research. Circulation Research, 47, 1-9.

Woody, C.D., and Brozek, G. (1969). Changes in evoked responses from facial nucleus of cat with conditioning and extinction of an eye blink. Journal of Neurophysiology, 32, 717-726.

Wurtz, R.H., Castellucci, V.F., and Nusrala, J.M. (1967). Synaptic plasticity: the effect of the action potential in the postsynaptic neuron. Experimental Neurology, 18, 350-368.